An Investigation of the Health Benefits of Honey as a Replacement For Sugar In the Diet

A thesis submitted in fulfilment
of the requirements of the degree of

Doctor of Philosophy

in Biological Sciences
at the University of Waikato
By

Lynne Merran Chepulis

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2008

Abstract:

Sugar (primarily sucrose) has been a part of the daily diet for literally hundreds of years, but research is now suggesting that sugar intake can be detrimental to our health. In particular, excessive consumption of simple sugars with high glycemic index (GI) values have been shown to cause overeating and weight gain.. As well, elevated postprandial hyperglycemia can result after consuming sugars and this has been linked to disease formation and progression, the development of advanced glycation endproducts, inflammation and increased mortality rates. Honey has been recognised as having a number of beneficial health properties, including slower uptake into the bloodstream, a pharmacological action of reducing blood glucose levels and a high level of bioavailable antioxidants, all of which may mean that honey could be less harmful to health than sucrose in the diet. This study was therefore designed to investigate the health benefits of honey in the diet as a replacement for sucrose, using small animal studies. As well, because of the interest in using honey as a replacement for sucrose in sweetened dairy foods, a small number of *in vitro* investigations were carried out to determine whether honey could retain its bioactive properties when combined with milk/dairy products.

Using the *in vitro* studies, it was shown that the combination of milk with honey had no effect on either the antibacterial or antioxidant capabilities of honey.

During the animal feeding studies a number of significant findings were observed. In the earlier work it was shown that honey had a significant effect on protein metabolism when fed for 14 days at a level of 600 g/kg diet (comprising 480 g sugars and 120 g water) compared with animals fed an equivalent amount of sucrose. In this study, honey-fed rats exhibited significantly lower weight gains ($p < 0.001$), food intake ($p < 0.05$) and nitrogen intakes ($p < 0.05$) and significantly higher faecal nitrogen outputs ($p < 0.05$) compared with sucrose-fed rats. Animals fed a diet consisting of 480 g/kg of mixed sugars as in honey generally exhibited protein metabolism parameters that were comparable to those of the sucrose-fed rats, suggesting that the effects of honey on protein metabolism were not due solely to its distinctive sugar composition.

Furthermore, in another study that specifically investigated the effects of honey on weight regulation, honey (100 g/kg diet) resulted in significantly reduced weight gain after 6 weeks ($p < 0.01$) compared with animals fed the same amount of sugars as sucrose, although food intake was not reduced in this study. Percentage weight gains were shown to be comparable between honey-fed rats and those fed a sugar-free diet, suggesting that differences in glycemic control may be partly responsible for the results seen. Fasting lipid profiles and blood glucose levels were

also measured in this study, but no significant differences were observed between diet groups.

During long-term (12 months) feeding weight gain was again significantly reduced in rats fed honey ($p < 0.05$) and a sugar-free diet ($p < 0.01$) compared with those fed sucrose, the weights of honey-fed rats and those fed the sugar-free diet being comparable at the end of the study. In addition, blood glucose levels were significantly lower ($p < 0.001$), and HDL-cholesterol levels significantly higher ($p < 0.05$) in animals fed honey compared with those fed sucrose after 52 weeks, but no differences in these parameters were observed between rats fed sucrose and a sugar-free diet. No other significant differences in lipid profiles were observed. Immunity measures were improved after feeding honey or sucrose for 52 weeks, animals in both of these diet groups having significantly higher levels of neutrophil phagocytosis compared with those fed the sugar-free diet (both $p < 0.0001$). In addition, the percentage of leukocytes that were lymphocytes was significantly higher in honey-fed rats at the end of the study. Furthermore, levels of oxidative damage in aortic collagen were significantly reduced in rats fed honey or the sugar-free diet (both $p < 0.05$) compared with those fed sucrose after 52 weeks.

Full body DEXA scans were also undertaken in this 12-month study to assess body fat levels and bone mineral composition and density, although they revealed few statistically significant differences. Percentage body fat levels were shown to be nearly 10% lower in honey-fed rats compared with sucrose-fed animals at the end of the study ($p < 0.05$), but no other significant differences between diet groups were observed. With one exception, no differences in bone mineral composition or bone mineral density were observed between the three diet groups after 52 weeks. This data agreed with the results generated from two earlier studies that showed that feeding honey short-term (for 6–8 weeks) to rats that were either calcium-deficient or fed a low calcium diet had no effect on bone calcium levels, bone mineral content, bone mineral density or bone breaking parameters.

Lastly, long-term feeding of honey to rats had a number of statistically significant effects on anxiety and cognitive performance when assessed using animal maze tasks. Anxiety-like behaviour was significantly reduced in honey-fed rats overall compared with those fed sucrose ($p = 0.056$) or a sugar free diet ($p < 0.05$). Spatial memory was also better in honey fed-rats throughout the 12 month study, these animals not displaying the same degree of age-related spatial memory loss seen in the other two diet groups. No significant differences in recognition memory or learning capability were observed between diet groups after 52 weeks.

In conclusion, both short-term and long-term feeding of honey result in a number of health benefits compared with eating similar amounts of sucrose. These

include less weight gain, improved immunity, reduced levels of oxidative damage and improved cognitive performance.. These effects of honey are likely to occur through a number of different processes, although the presence of high concentrations of antioxidants and other minor components in honey are likely to be important contributors. Honey may therefore help to improve human heath if it is used as an alternative to sucrose in foods and beverages, although feeding studies in humans are required to assess its efficacy. In addition, more animal studies are needed to assess which features of honey (*e.g.* fructose content, antioxidant content and bioactivities) are required to achieve optimal effects, and to determine what impact heating and food processing may have on the beneficial health effects of honey.

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To my baby girl, Sophie
May you live a long and happy life.
With all my love.

Abbreviations:

ABTS	2,2'-Azino-bis,3-ethylbenzthiazoline-6-sulphonic acid
AGE	Advanced glycation end-products
ANOVA	Analysis of variation
BMC	Bone mineral composition
BMD	Bone mineral density
CFR	Crop and Food Research
CHD	Cardiovascular heart disease
COV	Coefficient of variation
DEXA	Dual energy X-ray absorptiometry
DNA	Deoxyribose nucleic acid
DNI	Dietary nitrogen intake
EDTA	Ethylenediamine tetraacetic acid
EPM	Elevated plus maze
FCR	Food conversion ratio
FITC	Fluoroscein-isothiocyanate
FNO	Faecal nitrogen output
GE	Gross energy
GI	Glycemic index
HbA1c	Glycated haemoglobin
HCl	Hydrochloric acid
HDL	High-density lipoprotein (cholesterol)
HNE	4-Hydroxy-2-nonenal
LDL	Low-density lipoprotein (cholesterol)
MDE	Malondialdehyde
N	Nitrogen
NPU	Net protein utilisation
OD	Optical density
PBS	Phosphate buffered saline
PVC	Polyvinyl chloride
RDI	Recommended dietary intake
SCFA	Short chain fatty acids
SEM	Standard error of the mean
TEAC	Trolox equivalent antioxidant capacity
UNO	Urinary nitrogen output

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May you live a long and happy life.
With all my love.

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EDTA	Ethylenediamine tetraacetic acid
EPM	Elevated plus maze
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FITC	Fluoroscein-isothiocyanate
FNO	Faecal nitrogen output
GE	Gross energy
GI	Glycemic index
HbA1c	Glycated haemoglobin
HCl	Hydrochloric acid
HDL	High-density lipoprotein (cholesterol)
HNE	4-Hydroxy-2-nonenal
LDL	Low-density lipoprotein (cholesterol)
MDE	Malondialdehyde
N	Nitrogen
NPU	Net protein utilisation
OD	Optical density
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UNO	Urinary nitrogen output

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Chapter One:

Introduction and Literature Review

This thesis does not follow a traditional format with one introduction, one methods section and one results section *etc*, but instead consists of individual research chapters (one for each study undertaken), each with its own specific introduction, methodology, results and discussion. The studies were conducted using rats as a model for humans, and each chapter was designed to investigate specific health benefits of replacing sucrose in the diet with honey. A general introduction is given in this first chapter, and an overall discussion is provided in Chapter 11 to assess and evaluate the results brought together from the various studies undertaken in this thesis.

The term 'sugar' is referred to throughout this chapter, and it must be understood that this generally refers to the use of sucrose, or normal table sugar. In some instances, authors have used the term sugar without specifying which particular substance they are referring to (*i.e.* sucrose, glucose, fructose *etc*) and in these cases it is assumed that they are using the word sugar as an all-encompassing term. As sucrose is by far the most commonly used sugar in manufactured foods and home baking in New Zealand, this thesis centres primarily around looking at how honey may compare to sucrose in the diet.

In today's society, life seems to move at a significantly faster pace than it did 100, 50 or even 20 years ago. As a result, there have been several changes to the way we prepare and eat meals, and to which foods are available at our local supermarket. Nowadays, our food markets tend to be dominated by highly processed foods and ready-to-eat meals, and people often place very little thought into what the nutritional value is of the food that they are consuming.

Alongside this, however, there is an increasing awareness of the need for sustainable health, and many individuals are now becoming aware of the high levels of harmful food ingredients (including sugars – see below) that are being ingested as a part of the daily diet. As a result, much research is now focussed on the use of natural therapies and food products that can provide healthier alternatives without compromising on taste, and those that can confer more than simple nutrition.

One area that has become of increasing interest in recent years is in the use of honey as a nutraceutical. A large amount of published literature is currently available on the use of honey as a topical medicine (specifically its use in external therapies such as wound care), but there appears to be little, besides anecdotal evidence and folklore, to support its use as an internal remedy. However, there is substantial published literature indicating that honey consumed in the diet may be a healthier alternative to consuming sugar, primarily as it contains antioxidants and has a lower glycemic index. Thus, this study has been designed to investigate some of the health benefits that honey may have when it is incorporated into the typical everyday diet (*i.e.* how it may affect the body after being ingested and/or absorbed) and to determine whether health benefits result from replacing sugars in the diet with honey. That said, much of the research carried out to date has been from *in vitro* studies, and some of the evidence presented in this review is derived from studies that are not directly related to nutrition or internal health. This material will be included as it lends strength to the research provided by others, and gives a general overview of the particular bioactivities that honey possesses. Readers who are interested in the topical applications of honey are referred to the review articles published by Molan (2000, 2002, 2006).

1.1 THE USE OF SUGAR AND OTHER SWEETENERS IN FOODS

1.1.1 History of Sugar

The original use of sugar dates back over 9 000 years to the island of New Guinea with the discovery of the bamboo-like plant sugar cane, its inner pith

yielding a juicy substance that could be chewed or sucked to release the sweet sap inside (Macinnis, 2002). The world's scriptures also have limited references to "sweet cane" and Gautama Buddha specifically refers to the use of sugar in 568 BC (Macinnis, 2002). Armies of Alexander the Great reported the presence of sugar cane in India around 325 BC and it is thought that sugar (from sugar cane) was taken to Egypt and the African continent around this time (Mintz, 1986). During the Middle Ages the knowledge and use of sugar began to spread throughout Asia and Europe; however, it was not until the development of the African slave trade in the mid 1400s that the processing and ready accessibility of sugar was established (Macinnis, 2002).

By the start of the 20th century, the level of sugar consumption had increased dramatically, sucrose production having increased from 250 000 tonnes in 1880 to more than 16 million tonnes in the early 1900s (Mintz, 1986). Sugars such as glucose and sucrose became essential ingredients in British diets (Woloson, 2002), and a major food component in other cultures (Macinnis, 2002). From 1970 onwards, the use of sugars increased even further with the belief that high-fat diets were responsible for the increasing weight gains seen in Western populations (Astrup, 1999). Low-fat products quickly became available on the food market, but many of these were bolstered with additional sugar to maintain the flavour and mouth feel (Leveille, 1997).

However, with the realisation that the Western world is generally getting more overweight and less healthy overall, specifically with the increased incidence of Diabetes Mellitus and other diet-related disorders, the popularity of simple sugars is beginning to change. Dietary intake of high-sugar foods such as cakes, biscuits, jams and ice-creams is now peaking and/or decreasing in some groups (Woloson, 2002), although in the US, intake of sugars has continued to increase over the last 30 years (USDA database, 2005). As such, manufacturers are trying to influence the health (and preference) of the consumer by producing many of the sugar-sweetened foods and beverages produced in low-sugar or sugar-free forms. As well, both the medical community and the general public are becoming aware of the negative consequences that excessive sugar consumption can have. These issues are discussed in the following section.

1.1.2 Problems Associated with Sugar Intake

Limited reports dating as far back as the 1600 – 1800s document that sugar intake may have some negative implications on health (Macinnis, 2002); however, not until recent times has research specifically focussed on the effects

that sugar consumption can have on human health. Nowadays, it is well accepted that excess sugar intake (particularly sucrose and glucose) can contribute significantly to weight gain because of its high energy content and high glycemic index (GI) value (Willet, 1998). Older research reports that the simple sugars such as sucrose have fast-acting but short-lasting effects on blood sugar levels, with consumption of these sugars leading to a rapid increase in blood glucose levels, followed by a rapid plunge back to low blood glucose levels as large quantities of insulin are released (Rabinowitch, 1945). This pattern of blood glucose levels ultimately could lead to further hunger and overeating, which can, in turn, lead to excessive energy intake and weight gain. However, this rebound effect is now questioned in the current literature (Wolever et al., 2008).

Importantly, it has also been demonstrated that diets that contain substantial amounts of sugars (including simple sugars such as sucrose and glucose) can be detrimental to health because of elevated postprandial hyperglycemia. In particular, diets containing substantial quantities of these ingredients have been linked with formation of atherosclerosis, cancer and insulin resistance, as well as with increased rates of mortality and morbidity (see Section 9.1 for further details). Elevated blood glucose levels have also been associated with the formation of glycated proteins and advanced glycation endproducts (AGEs). These non-enzymatic adducts of proteins, lipids and nucleic acids (Bierhaus *et al.*, 2005) form spontaneously in pro-oxidant environments, the level of glycation depending on the degree and duration of hyperglycemia (Peyroux and Sternberg, 2006). AGE formation has been shown to contribute to the development and progression of a number of chronic disorders including hypertension, vascular disease and atherosclerosis (reviewed by Vasdev *et al*., 2007) and there is strong evidence to suggest that AGEs may potentiate the development of diabetic complications (Peppa and Vlassara, 2005; Ahmed and Thornally, 2006; Yamagishi *et al*., 2007). It has been suggested that the latter occurs by activation of cell responses by AGE-modified proteins interacting with specific cell surface receptors, impairments of protein-protein and enzyme-substrate interactions by AGE residue formation and increasing resistance to proteolysis of extracellular matrix proteins (Ahmed and Thornally, 2006).

Importantly, AGEs have also been shown to bind to RAGE (AGE receptor), a multi-ligand receptor of the immunoglobulin superfamily (Bierhaus *et al*., 2005). Engagement of RAGE acts to convert cellular function to sustained cellular dysfunction and tissue destruction, resulting in late diabetic complications such as neuropathy and nephropathy as well as macrovascular disease

(Bierhaus *et al*., 2005). Binding to RAGE also results in intracellular signalling which leads to activation of the proinflammatory responses (Bierhaus *et al*., 2001), thereby leading to chronic inflammation. Additionally, cellular inflammation can result in the formation of further reactive oxygen species which act to upregulate the proinflammatory response (Bierhaus *et al*., 2005. This self-amplification cycle then leads to further damage (Inflammation is discussed further in Section 1.5).

Thus, with the knowledge that excessive sugar intake can potentially be detrimental to health, there has been a move towards the development and discovery of alternative sweeteners. Today, a suite of substitutes is available to replace the more commonly used sugars (glucose and sucrose). These range from artificial sweeteners (see Section 1.1.3) to more complex sugars and plant sugars that are either sweeter or not absorbed, or they offer nutritional advantages in other ways (see Section 1.1.4). As well, foods that can offset some of the sugar-related damage are being investigated. These include foods that have a lower GI than sucrose and glucose (thereby leading to less hyperglycemia) as well as those that contain antioxidants (as antioxidants can help minimise the damage that results due to oxidative stress and reactive oxygen species formation from inflammation). Honey fits into both of these categories, and therefore offers the potential to provide both sweetness in foods and a protective effect after consumption. The bioactive properties of honey are discussed in Section 1.2.

1.1.3 Artificial Sweeteners

With the increased awareness of the risks associated with elevated levels of sugar intake, food manufacturers begun to look for alternative methods by which they could sweeten their products. The use of artificial sweeteners was established in the early 1970s, although their usage did not increase dramatically until the 1980s when it was found that sugars can substantially contribute to obesity and weight-related disorders. Currently there are four artificial sweeteners available on the market: saccharin, aspartame, sucralose and acesulfame potassium (Kandelman, 1997). Products containing these ingredients are worth at least 1.5 billion dollars per annum in the United States alone (Kinghorn *et al.*, 1998). Currently aspartame has the largest market share, as it is the major sweetener used in soft drinks. As well it has recently been approved as a “general-purpose sweetener” allowing it to be used in all types of food and beverages (Kinghorn *et al.*, 1998).

Despite the fact that the average intake of these sweeteners is below the recommended daily intake levels (set by the Joint FAO/WHO Expert Committee on Food Additives) (Renwick, 2006) there is now considerable debate over the safety of these sweeteners. A review of the literature suggests that whereas both aspartame and saccharin are generally safe and non-toxic when consumed in foodstuffs (Leon *et al.* 1989; Renwick, 1994; Ellwein and Cohen, 1990; Butchko *et al.* 2002); limited evidence is producing conflicting data. Several authors, for example, have reported a higher incidence of cancer in rats and mice fed sodium saccharin (Bryan *et al.,* 1970; Schoenig *et al.* 1985; Ellwein and Cohen, 1990; Rewick, 1990; Garland and Cohen, 1995; Gallus *et al.*, 2007). In addition, ingestion of aspartame has been linked with migraines/headaches (Lipton *et al.* 1989; van den Eeden *et al.* 1994; Schiffman, 1995), muscle tremors, vision problems and mental confusion (Anon, 1994).

Acesulfame potassium and sucralose have only been approved for use in foodstuffs in the United States since 1988 and 1998, respectively, and very little research has been undertaken to evaluate their toxicity and safety. Limited data suggests that sucralose may also trigger migraines in at-risk individuals (Patel *et al.*, 2006; Bigal and Krymchantowski *et al.*, 2007) although the same has not been reported with Acesulfame potassium. Furthermore, whilst no carcinogenic risks have currently been associated with the use of these new-generation sweeteners (Goldsmith, 2000; Mann *et al.*, 2000), it is generally accepted that it is too early to establish any epidemiological evidence about the possible carcinogenic risks (Weihrauch and Diehl, 2004).

Thus, with the possible negative connotations associated with the use of artificial sweeteners, there has been a drive towards the discovery of other, more natural, products that could potentially offer sweetness without harming health. In particular, increasing public awareness of obesity and the role that the additional calories of sucrose, glucose *etc* in foods and beverages can have has led to an impetus to discover products that may offer not only sweetness, but added benefits with respect to calorie intake, weight regulation and overall health.

1.1.4 Natural Sweeteners

In recent years much research has been undertaken to assess the various sources of sweet-tasting compounds in the plant kingdom, and, to date, more than 75 highly sweet compounds have been identified (Kinghorn *et al.*, 1998). These compounds consist mainly of terpenoid, flavonoid and protein

compounds, although nine different structural groups of potentially sweet molecules have bee documented overall (Kinghorn *et al.*, 1998).

A small number of plant-based sweeteners are now being used commercially as sucrose substitutes, the most common of which are the steviol glycosides stevioside and rebaudioside A, from the South American plant *Stevia rebaudiana* (Das *et al.*, 1992). Other plant-derived sweeteners approved for use include glycyrrhizin (from the roots of *Glycyrrhiza glabra L.*) and the protein thaumatin from fruits of *Thaumatoccus daniellii* (Kinghorn *et al.*, 1998). As well, many kinds of sweet cucurbitane glycosides have been isolated from *Cucurbitaceous* plants and from the fruits of *Momordica grosvenori* (Cucurbintaceae) (Takasaki *et al.*, 2003). Limited data is available on the safety of these compounds as food additives, although most of these compounds have been given GRAS (Generally Regarded as Safe) status in the United States. Several of these compounds may offer other advantages as well. Stevioside and extracts of *Momordica grosvenori*, for example, have been shown to have anticarcinogenic properties *in vitro* (Takasaki *et al.*, 2003).

The above plant-based sweeteners are often also known as “intense” sweeteners as they offer a sweetness intensity that is greater than that of sucrose (Kinghorn *et al.*, 1998). However, there are also other sweeteners, termed “bulk” sweeteners, that are less sweet that sucrose. These compounds include isomalt as well as the sugar alcohols sorbitol, xylitol, lactitol and mannitol (Kinghorn *et al.*, 1998). Bulk sweeteners have been shown to offer a number of nutritional advantages over the use of sucrose and simple mono- and disaccharides. Sugar alcohols, for example, are not readily fermented by oral bacteria, so they may aid in reducing the development of sugar-related dental caries (Kandelman, 1997). As well, research in animal models has shown that xylitol and sorbitol may aid in the absorption of calcium from the gut, this occurring through the complexing of calcium in the intestine (see Chapter 7).

Whereas these natural sweeteners offer a good alternative to the use of more mainstream sugars such as sucrose and high fructose corn syrup, as well as the artificial sweeteners used in diet products, the methods of extraction and the often small quantities produced can make such natural sweeteners prohibitively expensive. This tends to restrict their use in large scale food production, and instead they are often viewed as luxury food additives that must be incorporated into food at the level of the consumer rather than at the level of the food producer. For this reason, the search is still on for food ingredients that are cost-effective to produce, whilst offering health advantages to the consumer,

particularly when incorporated into mainstream food production. Several large food production companies are currently looking at ways to improve the "healthiness" of their products, and as a result, products such as cholesterol-lowering spreads are now reaching the supermarket shelves, albeit at premium prices.

Honey is a natural sweetener that has been used since ancient times (see Section 1.2.1) and its use as a food ingredient in products such as spreads and marinades is well established. Only in recent times, however, have the potential health benefits of honey begun to be explored. It is now recognised that honey contains a mix of both simple and complex sugars, thereby providing good levels of sweetness, as well as vitamins, minerals, acids and enzymes (Molan, 1996). Furthermore, it has been demonstrated in animal and clinical studies to have several health-promoting and medicinal properties. These will be discussed, in detail, in the following sections.

1.2 HONEY: AN OVERVIEW

1.2.1 Historical Uses of Honey

Honey has long been documented as having medicinal properties, and its uses as a wound dressing and an antiseptic have been recorded since ancient times. The earliest written records of honey used as medicine is in Egyptian papyri and Sumerian clay tablets dated from 1900 to 1250 BC and in one of these, honey was used in 30% of the prescriptions (Stomfay-Stitz, 1960). Ancient Egyptians also used honey in embalming. They made salves with it for treating diseases of the eyes and skin (Al Waili, 2003a). As well, the ancient Greeks are reported to have used honey to treat fatigue: athletes drank a mixture of honey and water before major athletic events (Wilson and Crane, 1975). Hippocrates (460-357 BC) found that honey cleaned sores and ulcers of the lips and healed buncles and running sores. As well, the healing properties of honey were mentioned in the Holy Quran 1400 years ago (Al Waili 2003a).

Honey has continued to be used in folk medicine ever since, with mention of its use in the Middle Ages reported by Daude de Pradas in approximately 1200 AD (Wilson and Crane, 1975). Similarly, honey has been documented as being used as a remedial agent throughout Europe, as well as through areas of Arabia and China (Beck and Smedley, 1997). Generally, honey has been used as a remedy for gastric and intestinal complaints, although the sedative and soporific powers of honey have also been mentioned (Beck and Smedley, 1997). In addition, the diuretic effect of honey has been recorded, and it has been a

favoured remedy for kidney inflammations and stones (Beck and Smedley, 1997). Attic honey, in particular, was thought to have special curative powers for eye disorders, and honey, in general, has been documented as having being used for the treatment of skin diseases and smallpox, as well as in surgical dressings (Beck and Smedley, 1997).

The Hindu people also had great faith in the medical virtues of honey, using it mainly for coughs, pulmonary issues and gastric disorders (Beck and Smedley, 1997). Similarly, populations in rural communities from almost all nations have documented the use of honey through time (Beck and Smedley, 1997). German women, specifically, believed that a mixture of honey and crushed bees would have a beautifying and strengthening effect, and that it would regulate menstrual flow (Beck and Smedley, 1997).

In more recent times, honey has played a relatively minor role in medicine, mostly due to it not being accepted by Western practitioners in a world where antibiotics and other pharmaceuticals are seen as the remedies of choice. Among the Chinese, Hindu, Arabic and African races, however, honey is still considered to be a valuable internal and external remedy (Beck and Smedley, 1997). Slowly the use of honey in Western medicine is gaining recognition; particularly as scientific evidence continues to be produced demonstrating its efficacy, often in situations where more usual remedies are ineffective. The antibacterial properties of honey (see Section 1.3) and its wound-healing capabilities (reviewed by Molan, 2006), in particular, have gained substantial recognition in the last 10–15 years, although only now are researchers beginning to understand the processes by which this occurs. We are also beginning to understand that honey may indeed be the elixir that the ancient people believed, as research is showing a number of health-related benefits, including a laxative effect, beneficial effects on blood glucose levels, anti-inflammatory and immune-stimulating properties and potentially a cancer-preventative action (see below).

1.2.2 Composition of Honey

Honey is primarily a highly concentrated mixture of sugars in a syrupy solution, although it usually also contains other minor components including enzymes, vitamins, flavanoids and organic chemicals (Molan, 1996).

The main sugars found in honey are fructose and glucose, usually in a ratio of 1.2:1.0, these two sugars together accounting for 85–95% of the total carbohydrate content (White, 1975a). However, the ratio of these two sugars can differ depending on the floral source and other factors such as the level of

processing undertaken by the bees. Other sugars found in minimal quantities include sucrose, reducing disaccharides and higher oligosaccharides (White, 1975b; Astwood *et al*., 1998; Sanz *et al*., 2004). The number of different oligosaccharides found in honey seems to vary depending on the floral source, although numbers of between 20 and 30 have been reported previously (Siddiqui, 1970; White, 1992).

Honey also contains a number of enzymes including glucose oxidase, amylase and invertase, their presence in honey appearing to originate from the bees producing the honey (Molan, 1992). Catalase (Dustmann, 1971) and acid phosphatase (White, 1975b) have also been found in some honeys, these most likely being derived from the pollen and nectar of certain plants. Of these enzymes, glucose oxidase appears to be of particular significance as it is responsible for the generation of gluconic acid and hydrogen peroxide (see Section 1.3.1), hydrogen peroxide being one of the key factors involved in the antibacterial activity of honey (see Section 1.3.2). Other acids, besides gluconic acid, are also present in honey (albeit in low concentrations), the overall result being that undiluted honey typically has a pH in the range of 3.2–4.5 (White, 1975a).

Additional research has also demonstrated that certain honeys can also contain low levels of minerals, trace elements and proteins (White, 1975a), although their nutritional significance is likely to be negligible due to their low concentrations (Haydak *et al.*, 1975). However, anecdotal and scientific evidence suggests that honey may also contain small quantities of unidentified but physiologically active components. For example, it has been suggested that something in honey may help suppress coughing, aid in desensitising pain receptors, have a laxative effect (Ladas *et al.,* 1995) and help with lowering blood glucose levels (Al Waili, 2003a, b). As well it has been documented as possibly having neurotransmitter activities (Goldschmidt and Burkert, 1955).

Generally, honey is considered to be a stable substance (White *et al.*, 1960) although changes in colour and flavour can occur over time. Minor changes in composition can also occur in certain honeys due to the continuing action of enzymes. The sucrose content, for example, has been shown to decrease over time (Browne, 1908; cited in White *et al.*, 1960), this being attributed to the activity of invertase added by the bee.

1.3 THE ANTIMICROBIAL ACTIVITY OF HONEY

Although the medicinal uses of honey have been documented for centuries, it has only been in recent times that the specific bioactivities have been investigated and elucidated. Of these, the antibacterial activity of honey is the most researched and best understood.

The significance of the antibacterial activity of honey relates more to its external uses as a wound dressing or skin-care agent, although it may also play a role in maintaining the balance of internal microflora after consumption. As well, honey has been shown in one clinical trial to be effective against bacterial diarrhoea, (Haffejee and Moosa, 1985), and to aid in the treatment of eye infections (Fotidar and Fotidar, 1945, cited in Molan, 2001; Al Waili, 2004a). In addition, the hydrogen peroxide produced by honey may have additional actions in the body besides its antibacterial activity. For these reasons, the antibacterial activity will be summarised below, although readers are directed towards the review of Molan (1992) for more detail.

1.3.1 Osmolarity and Acidity

The antimicrobial activity of honey is due to a number of factors, several of which are endemic to all or most honeys, irrespective of floral source and geographic origin. The first of these is osmolarity. Because of the high sugar content of honey, the osmotic pressure of honey is high and the water activity (a_w) is low (reported range = 0.562–0.62; Tysset *et al.*, 1980; Ruegg and Blanc, 1981; Bogdanov *et al.*, 1987). Osmolarity is responsible for a large proportion of the antimicrobial activity of honey, even at lower honey concentrations. The growth of many bacterial species, for example, is completely inhibited when the a_w is in the range of 0.94-0.99 (Scott, 1957; Leistner and Rodel, 1975). This corresponds to a typical honey (with an a_w of 0.6) being diluted in the range of 2–12% (concentration is proportional to $-\log a_w$; Scott, 1957).

The second factor common to all honeys is acidity. Approximately 30 organic acids exist in honey (Mato *et al.*, 2003), although the major contributor is gluconic acid in equilibrium with its lactone. This acid is produced by the activity of the enzyme glucose oxidase, the resultant concentrations in honey ranging from 0.23–0.98% (White, 1975a) (See Figure 1.1).

Although once thought to be a major factor, more recent studies have shown that acidity actually plays a very small role in the antibacterial activity of honey (Pothmann, 1950, cited in Molan, 1992). Several authors noted that there appeared to be no correlation between the pH of a honey and the level of

antibacterial activity (Stomfay-Stitz *et al.*, 1960; Rychlik and Dolezal, 1961; Lindner, 1962; Daghie *et al.*, 1971; Ruegg and Blanc, 1981; Bogdanov *et al.*, 1987). This may be confounded by the fact that the pH of a diluted honey is dependent on both the buffering capacity of the honey and on the dilution medium used.

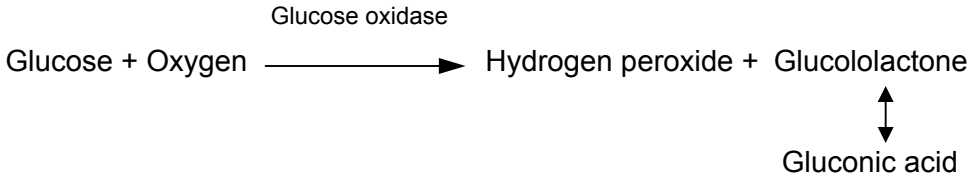

Figure 1.1: *Production of hydrogen peroxide and gluconic acid by the enzyme glucose oxidase.*

In contrast a small number of research articles have suggested that certain specific acid substances may have significant antibacterial activity (Russell *et al.*, 1990; Wahdan, 1998; Weston *et al.*, 1998). Acid components identified as antibacterial are mainly aromatic organic acids including caffeic acid and ferulic acid (Wahdan, 1998) and benzoic acid derivatives (Russell *et al.*, 1990; Weston *et al.*, 1998). It must be noted though that both the studies of Russell *et al.* (1990) and Weston *et al.* (1998) looked solely at Manuka honey, and is unknown whether these compounds occur in honeys originating from other floral sources.

1.3.2 Hydrogen Peroxide

Hydrogen peroxide was shown to be a significant contributor to the antimicrobial activity of honey when Dold *et al.* (1937) found that certain honeys exhibited inhibitory activity over and above that expected from osmolarity alone.

It has also been demonstrated that hydrogen peroxide can have a more potent antibacterial activity when present in honey than when added exogenously (Molan, 1992), as there are other compounds present in honey that can potentiate its antibacterial effects. The bactericidal action of hydrogen peroxide, for example, is increased when 0.1 mmol/l ascorbic acid and metal ions are

added to the test solution (Miller, 1969; Roos and Balm, 1980). Similarly, the sporicidal action of hydrogen peroxide can be increased by copper at 10 mmol/l (Waites *et al.*,1979). The antibacterial potency of hydrogen peroxide is also enhanced 10-fold by the addition of 0.83 mmol/l iron, copper, chromium, cobalt or manganese (McCulloch, 1945). All of these ions occur naturally in honey (albeit in lower concentrations than those reported above; White, 1975a) suggesting that maximal rates of hydrogen peroxide-induced antibacterial activity can occur when using honey as the antibacterial agent.

1.3.2.1 Variability of Glucose Oxidase Activity / Hydrogen Peroxide Production

Glucose oxidase activity (and therefore hydrogen peroxide production) varies substantially in different honeys, with some honeys showing no activity at all. Generally, low concentrations of hydrogen peroxide (and/or low levels of hydrogen peroxide-induced antimicrobial activity) are correlated with low levels of glucose oxidase activity and vice versa. However, this is often an incorrect assumption, as the lack of observed activity may be a result of hydrogen peroxide destruction rather than a reduction in glucose oxidase activity.

The low levels of glucose oxidase activity seen in some honey samples may, in part, be the result of loss of activity during processing and handling of the honey by the apiarists. The glucose oxidase enzyme that generates hydrogen peroxide when honey is diluted is denatured by heat (White and Subers, 1964a, White, 1966; Bogdanov *et al.*, 1987) and by light (Duisberg and Warnecke, 1959, White and Subers, 1964b; Dustmann, 1979). In a small study using only 13 honey samples, Revathy and Banerji (1980) demonstrated that processing raw honey before marketing did reduce its antibacterial activity. However, in a larger study by Allen *et al.* (1991) involving 345 different samples, no correlation was found between age, extraction, storage and processing with the levels of antibacterial activity.

The level of hydrogen peroxide production may also be affected by the floral source as Allen *et al.* (1991) demonstrated that many dark coloured honeys were often associated with high levels of hydrogen peroxide-induced antibacterial activity whereas light coloured clover honeys were consistently found to have low activity. In addition, particular floral sources have been shown to contain high levels of catalase, a hydrogen peroxide degrading enzyme, such that the overall level of accumulation is low (Schepartz, 1966; Schepartz and Subers, 1964;

Dustmann, 1979). Alternatively, honeys with a low level of catalase may accumulate high levels of hydrogen peroxide.

However, not all variation of hydrogen peroxide by floral source is due to the presence of catalase. It has been found that hydrogen peroxide added to honey will be destroyed even if the honey has been heat-treated beforehand such that no catalase is active, indicating that chemical degradations are involved as well as enzymic destruction (White *et al.*, 1963). Molan (1992) suggested that this could be due to metal-catalysed reaction with ascorbic acid.

It is also possible that either glucose oxidase and/or the hydrogen peroxide it produces may be prone to destruction/inactivation by other components of honey. White *et al.* (1963) found that glucose oxidase is virtually inactive in full strength honey but that activity increasing 2500–50000 times when the honey is diluted. This data suggests the presence of a potent enzyme inhibitor(s) in the honey. These inhibitory compound(s) have largely not been investigated, nor have they been identified, however, it is highly possible that the factors responsible may vary in different honeys.

1.3.2.2 Rates of Hydrogen Peroxide Production in Honey

The ability to accurately quantify glucose oxidase activity, or more importantly, the inherent level of hydrogen peroxide produced/accumulated in a given honey sample depends largely on the methodology employed. There are many different methods available for the measurement of hydrogen peroxide (Welcher, 1963; Parker, 1978; Jie *et al.*, 1995; Bang, 1998), but whereas these methods produce reliable and accurate results with standard water-based solutions, they have been shown to not accurately detect and measure hydrogen peroxide in honey due to various side reactions (Bang, 1998).

Recently, an assay not based on the use of peroxidase was developed using an oxygen electrode to fully elucidate the hydrogen peroxide production profile in different dilutions of honey (Bang *et al.*, 2003). These authors demonstrated that this method was not prone to interference from other components of honey, and that hydrogen peroxide levels could be measured in honey concentrations of up to 80%. Various single- and multi-floral honeys were assayed in this study (including Ling Heather, Rewarewa, Clover, Wildflora, Bush and Pasture) and the profiles were primarily the same for all samples. Hydrogen peroxide production ranged from 0.3–1.4 mmoles/30 mins in 10% dilutions of honey, to 0.9–2.9 mmoles/30 mins in 30% solutions. Peak rates of production occurred at 30–40% honey and, in all samples, the rates of hydrogen peroxide

production were virtually the same at 10% and 70% honey dilutions. Levels of accumulated hydrogen peroxide over time ranged between samples. Peak levels were accumulated after 6 and 24 hours, respectively, in a Ling Heather and Rewarewa sample, with total loss of all detectable hydrogen peroxide after 24 and 48 hours (Bang *et al.*, 2003).

1.3.3 Other Components

Following the discovery that the antibacterial activity of honey was primarily due to hydrogen peroxide, it was suggested that non-peroxide factors in honey likely play a minor role in the antibacterial action (Dustmann, 1979; Morse, 1986; White and Subers, 1963). However, a number of researchers demonstrated the presence of non-peroxide antibacterial factors when they found that particular honeys exhibited an antibacterial activity even after the honey had been heat-treated (Gonnet and Lavie, 1960; James *et al.*, 1972; Roth *et al.*, 1986; Molan and Russell, 1988) or after treatment with catalase to remove the hydrogen peroxide (Adcock, 1962; Roth *et al.*, 1986; Molan and Russell, 1988; Hodgeson, 1989; Allen *et al.*, 1991).

In recent times, much research has focussed on the non-peroxide antimicrobial activities of New Zealand Manuka (*Leptospernum scoparium*) honey. Analysis of an ether extract of Manuka honey demonstrated that Manuka honey contained components with antibacterial activities including syringic acid, methyl syringate and phenolic acids (Russell *et al.*, 1990) and these authors suggested that these compounds may be responsible for the non-peroxide activities observed in this type of honey.

As well as the non-peroxide antimicrobial properties of Manuka honey, certain honeys have also been shown to contain a number of other antibacterial compounds. These include the plant-derived flavonoids pinocembrin, pinobanskin and chrysin (Bogdanov, 1984; Ferreres *et al.*, 1994; Soler *et al.*, 1995) and the phenolic compounds caffeic acid and ferulic acid (Bogdanov, 1997; Wahdan *et al.*, 1998; Weston *et al.*, 1998; 2000). As well, Mundo *et al.* (2004) reported that Manuka, Soybean, Black Sage and Blackberry honeys demonstrated non-peroxide inhibition of various bacteria that was reversed in the presence of α-chymotrypsin, indicating that proteinaceous compounds may also be involved in the antibacterial properties of some honeys. These protein-based compounds have yet to be elucidated.

1.4 ANTIOXIDANT CAPACITY OF HONEY

1.4.1 Oxidative Stress

Oxidative stress is defined as the imbalance between free radical production and the antioxidant defense system resulting in an excess of oxidation (Gheldof and Engeseth, 2002). Oxidant free radical molecules are produced during normal metabolism in the cell and evidence now suggests that the excessive generation of these molecules can lead to DNA damage (Holmes *et al.*, 1992; Ames *et al.*, 1993) and contribute to the development of certain age-related pathologies including arthritis, strokes, atherosclerosis and some cancers (Peto *et al.*, 1981; Cross *et al.*, 1987; Schramm *et al.*, 2003). Antioxidants are compounds that can reduce levels of oxygen free radicals, thus countering their toxic effects. They can occur endogenously within the body or be provided by dietary sources. There is a large amount of evidence in the literature to suggest that consumption of foods, in particular fruit and vegetables, can boost levels of antioxidants thereby playing a significant role in reducing the incidence of cerebovascular and other diseases (Machlin 1995; Steinmetz and Potter, 1996; Van Duyn and Pivonka, 2000). The process of oxidative stress and the role of antioxidants is discussed further in Chapter 10.

1.4.2 Antioxidants in Honey

Within the last 10 years, several studies have shown that honey does have an antioxidant capacity *in vitro* (See Table 1.1). Furthermore, honey has been shown to prevent lipid oxidation in cooked ground turkey meat (Mathew *et al.*, 1998; Antony *et al.*, 2000; McKibbon and Engeseth, 2002), with honey concentrations as low as 5% significantly reducing lipid oxidation by approximately 70% after 1–3 days. In addition, honey (5%, w/w) was more effective than α-tocopherol and butylated hydroxytoluene, two commonly used phenolic antioxidants, after both 1 and 3 days (McKibbon and Engeseth, 2002).

Honeys from different floral sources have also been shown to inhibit the enzymatic browning of fruit and vegetable homogenates (Oszmianski and Lee, 1990; Chen *et al.*, 2000). In the latter study, honeys reduced the browning of baking potato, sweet potato, red delicious apple and d'Anjou pear by up to 45%, but it was less effective than the commercial browning inhibitors ascorbic acid and sodium metabisulphate.

Table 1.1: *Antioxidant content of honey*

Reference	No. samples tested* (no. different floral sources)	Average Antioxidant Level	Method of Measurement
Frankel *et al.*, 1998	19 (14)	21.3 – 432 x 10^{-2} μeq/g	DPPH
Chen *et al.*, 2000	7 (7)	2.5 – 59.2 x 10^{-1} μeq/g	TBARS
Gheldof and Engeseth, 2002	14 (7)	3.1 to 16.3 μmol TE/g	ORAC

* excluding sample replicates, DPPH = 1,1-diphenyl-2-picrylhydrazyl, ORAC = oxygen radical absorbance capacity, TBARS = Thiobarbituric acid reactant substances,TE = trolox equivalents

More recently, a small number of studies have investigated the *in vivo* antioxidant capacity of honey (Gheldof *et al.*, 2003; Busserroles *et al.*, 2002; Schramm *et al.*, 2003; Perez *et al.*, 2006). Gheldof *et al.* (2003) found that the serum antioxidant capacity, measured using oxygen radical absorbance capacity (ORAC), was increased by 7% in individuals who had consumed a 500 ml solution of Buckwheat honey (160 g/l), but not in those who consumed 500 ml of black tea or black tea plus 160 g/l of a syrup of mixed sugars (45% fructose, 35% glucose, 20% water). Similarly, in another study, plasma antioxidant capacity increased by 12–25% six hours after the ingestion of a single dose of Buckwheat honey (1.5 g/kg body weight), and the plasma total-phenolic content was increased by 4–8% (Schramm *et al.*, 2003). No significant effects on either parameter were reported in subjects who consumed an equivalent amount of corn syrup (Schramm *et al.*, 2003). The plasma reducing capacity was also shown to be improved by approximately 10% following ingestion of honey (Schramm *et al.*, 2003). In a 2-week rat feeding study in which animals were fed diets containing 65 g of carbohydrate (starch, honey or a mixture of fructose and glucose as in honey) per 100 g of diet, honey-fed rats had higher plasma α-tocopherol levels, lower plasma nitrogen oxide levels and a lower susceptibility of lipid peroxidation in the heart (Busserroles *et al.*, 2002). In contrast, pro-oxidative effects were observed in rats fed the fructose/glucose mixture.

The antioxidant capacity of honey has been attributed to several factors including α-tocopherol, polyphenolics, organic acids, ascorbic acid, β-carotene and enzymes (Crane, 1975; Gheldof *et al.*, 2003). In particular, research has

suggested that the antioxidant capacity of honey is largely due to its total phenolic content (Gheldof and Engeseth, 2002; Gheldof *et al.*, 2002), and several studies have demonstrated that many honeys do have a rich phenolic profile consisting of benzoic acids and their esters, cinnamic acids and flavonoid aglycones (Ferreres *et al.*, 1992, 1994; Andrade *et al.*, 1997; Martos *et al.*, 2000; Gheldof *et al.*, 2002). In one study (Gheldof *et al.*, 2002) a chromatographic analysis of the phenolic fractions of 8 honeys of different floral sources suggested that most honeys have quantitatively different phenolic profiles, and that a linear correlation exists between phenolic content and ORAC activity (Gheldof *et al.*, 2002). This same study reported that whereas phenolic compounds contributed significantly to the antioxidant capacity of honey, they were not solely responsible. These authors also found a positive correlation between protein content and ORAC activity and acknowledged that other antioxidant components were also involved. Several authors have also reported that there is a significant positive correlation between antioxidant capacity and honey colour, the darker coloured honeys having higher antioxidant activities (Frankel *et al.*, 1998; Taormina *et al.*, 2001; Gheldof and Engeseth, 2002). Whether this is due to different phenolic profiles in the different coloured honeys, or due to other floral factors is unknown. In addition, Frankel *et al.* (1998) reported that the antioxidant capacity of honey was directly correlated to the water content.

Today there is reasonable interest in the use of honey in foodstuffs, and one particular area of interest lies in its ability to offer an antioxidant effect although there are contrasting data as to whether the antioxidant capacity of honey is comparable to other food sources. Frankel *et al.* (1998), using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method, reported that antioxidant levels in honey (average 0.9×10^{-3} μeq/g) tended to be lower than the more traditional sources of dietary antioxidants including sweet orange pulp (5.7×10^{-3} μeq/g), broccoli (13.6×10^{-3} μeq/g) and sweet peppers (14.2×10^{-3} μeq/g) as assayed by Sebrell *et al.* (1967) using the same methodology. In contrast, the antioxidant content of honey determined using the ORAC assay (3–16 μmol trolox equivalent (TE) units/g) by Gheldof and Engeseth (2002) were in the same range as many fruits and vegetables measured using the same assay (0.5–19 μmol TE/g fresh weight; Cao *et al.*, 1996; Wang *et al.*, 1996). It is important to note, though, that honey is generally not consumed in the same quantities as most fresh fruit and vegetables. More likely it is used as a healthy alternative to sugar, and consumed in subtantially smaller portions than other dietary antioxidant sources, thereby

serving as a supplementary source of antioxidants. However, if even some of the sugar in the typical diet were to be replaced with honey, this could result in a significant increase in the intake of antioxidants.

1.5 ANTI-INFLAMMATORY ACTIVITY OF HONEY

1.5.1 Significance of Inflammation in Health

Inflammation is an important component of the normal reaction to infection or injury involving a series of inter-related events between immune cells, phagocytes, cytokines and other inflammatory mediators (Wan *et al*., 1989). However, when it occurs in excessive amounts or for prolonged periods of time it can actually be detrimental, preventing healing from occurring or even causing further damage (Molan, 2001).

The most serious consequence of inflammation is the production of oxidant free radicals in the tissues (Flohe *et al*., 1985) (refer to Sections 1.4.1 and 9.1 for a discussion on oxidative stress). This, in turn, can lead to the breakdown of lipids, proteins and DNA, thereby resulting in loss of cell functioning and tissue damage (Cochrane, 1991). In certain situations, reactive oxygen species can be produced in excessive amounts. During phagocytosis, for example, H_2O_2 and $O_2^{\bullet-}$ are produced and these recruit more lymphocytes, thereby creating a self-amplification cycle (Flohe *et al*., 1985). As well, reactive oxygen species can activate tissue proteases which causes greater damage than just that due to the free radicals formed from the H_2O_2 and $O_2^{\bullet-}$ (van Kempen *et al*., 2006).

Importantly, there is now evidence to suggest that there are strong correlations between inflammation and many health disorders. Chronic inflammation, for example, is now considered to be a major contributing factor in several diseases including arthritis, cancer and inflammatory bowel disease (Wan *et al*., 1989). As well, systemic inflammation has been shown to either initiate or worsen autoimmune disorders (including type I Diabetes), pre-senile dementia (Alzheimer's disease) and atherosclerosis (reviewed by Brod, 2000). The formation of atherosclerosis, in particular, is a particular consideration in this thesis as Diabetes is a major risk factor for its formation because of hyperglycemia-induced endothelial dysfunctions (Mitai and Agrawal, 2007). However, whilst several studies have demonstrated that inflammation is a key part of atherogenesis (Stoll and Bendszus, 2006; Mitai and Agrawal, 2007; Singh and Devaraj, 2007) researchers are now suggesting that nutrition may be used as an intervening factor (Wan *et al*., 1989; Paoletti *et al*., 2006).

Recent data from large clinical studies has also demonstrated that there is an association between inflammation and neuropsychiatric symptoms seen in aging, with elevated circulating levels of C-reactive protein, interleukin-6, interleukin-10, tumour necrosis factor and serum amyloid A (markers of inflammation) correlating with reduced cognitive function in older age (Dimopoulos *et al*., 2006; Jordanova *et al*., 2007; Schram *et al*., 2007). This is also an important factor to consider in this thesis as many of the impairments of cognitive function are known to occur because of oxidative stress in the brain (see Section 10.1 for details) and honey is known to be a strong antioxidant agent (see Section 1.4.2).

It has recently been suggested that obesity may also be a chronic inflammatory illness, as there is an increase in inflammatory markers in the circulation of obese individuals, and macrophages have been identified in their white adipose tissue (Cancello and Clement, 2006). This too, may be significant to consider in relation to this thesis as the different effects of sucrose and honey on weight regulation are an important measure in several studies in this thesis (see Chapters 5, 6 and 9).

1.5.2 The Anti-Inflammatory Activity of Honey

Although it is possible that honey may have an anti-inflammatory effect after consumption, most of the research into this area has primarily centred around the use of honey as a topical agent. Numerous studies, for example, have reported that honey applied to wounds reduces the localised swelling, heat and pain associated with inflammation (Burlando, 1978; Efem, 1993; Subrahmanyam, 1993, 1998).

Though the process by which honey acts as an anti-inflammatory agent has yet to be fully elucidated, the effect has been widely observed clinically (Molan, 2006). Manuka honey incorporated in a gel for topical use was shown to suppress the itching and erythema associated with mosquito bites and other inflammatory skin reactions due to local anaphylactic reactions (P. C. Molan; personal communication). These symptoms are produced by the increased liberation of prostaglandins resulting in development of localised hot areas (Dascombe, 1985). This indicates that honey acts to decrease prostaglandin synthesis. Indeed, in one small clinical study (n=12) consumption of honey was shown to have significant effects on urinary total nitrite and prostaglandin concentrations (Al-Waili, 2005). In this study, patients were given a solution of either honey (80 g in 250 ml water) or artificial honey (30 g sucrose and 38 g

fructose in 250 ml water). Urinary prostaglandin E2 levels were decreased by 4%, 6% and 31%; prostaglandin F2-α by 15%, 28% and 44% and thomboxane B2 by 15%, 36% and 67% at 1, 2 and 3 hours after drinking the honey. In contrast, ingestion of artificial honey increased these parameters at most time intervals.

Honey has also been observed to reduce inflammation when applied to unbroken skin, indicating that the anti-inflammatory activity may be able to diffuse through the skin (Molan, 1992). Furthermore, honey decreased the stiffness of inflamed wrist joints in guinea pigs (Church, 1954), a standard test for anti-inflammatories.

Importantly, it has also been shown in histological studies of wounds in both animals and humans (Kandil *et al*., 1987; El-Banby *et al*., 1989; Cochrane, 1991; Gupta *et al*., 1992; Oryan and Zaker, 1998) that honey is effective even when there is no infection present. This suggests that the anti-inflammatory effect of honey is a direct effect, and not a secondary effect resulting from the removal of inflammation-causing bacteria.

1.5.2.1 Are the Anti-Inflammatory Properties of Honey due to its Antioxidant Content?

As mentioned above, the most serious complication of excessive inflammation is the production of reactive oxygen species in the tissue, these being produced as side products of phagocytic activity in the inflammatory process (Flohe *et al*., 1985). Importantly, though, reactive oxygen species also recruit more leukocytes into areas of inflammation which leads to self-amplification of the inflammatory response via oxidative activation of the transcription factor NF-κB that promotes the production of pro-inflammatory cytokines (Flohe *et al*., 1985). This, in turn, can stimulate fibroblasts as well which can, if present in excessive amounts, lead to fibrosis (scarring and adhesions) (Molan, 2001).

It has been suggested, therefore, that the antioxidant content of honey may account for its anti-inflammatory activity as the activation of NF-κB can be prevented by antioxidants (Grimble, 1994), and direct application of antioxidants to burns has been demonstrated to reduce inflammation (Burlando, 1978; Subrahmanyam, 1991). Secondly, honey is likely to prevent the feed-back amplification of inflammation that results due to hydrogen peroxide. Clinical studies have confirmed that hydrogen peroxide is directly inflammatory to skin and this is why it is no longer used as an antiseptic on tissues (Saïssy *et al.*,

1995; Halliwell and Cross, 1994; Salahudeen *et al.*, 1991; Lineaweaver *et al.*, 1985). Antioxidants have been demonstrated to block the stimulatory effects of hydrogen peroxide and to prevent the oxidation of regulatory proteins which occurs when free radicals form from it (Grimble, 1994). It is important to note, though, that most honeys endogenously produce hydrogen peroxide (when they are diluted) as well as potentially blocking its stimulatory effects and oxidant production. However, production of hydrogen peroxide by glucose oxidase in honey requires a neutral pH and a good supply of oxygen (Schepartz and Subers, 1964), and neither of these conditions are readily met in the stomach or intestinal tract.

As well, research has also provided evidence that antioxidants have potent effects on immune cell function (Bourdon *et al.*, 1999; Grimble, 1994; Ames *et al.*, 1993) and that the ability of other bioactive agents to suppress inflammation is based on their antioxidant content (Basova *et al.*, 2002; Meydani and Erickson, 2001; Alliangana, 1996; Rao *et al.*, 2003). This adds weight to the suggestion that the antioxidant content of honey may be responsible for it having an anti-inflammatory effect, though there have been no studies to date to confirm this hypothesis. However, in one study that showed that topical administration of honey can reduce post-operative peritoneal adhesions, it was suggested that this was due to its content of caffeic acid, benzoic acid, phenolic acid, and flavonoid glycones (Aysan *et al.*, 2002).

1.6 PREBIOTIC EFFECT OF HONEY

1.6.1 Probiotics and Prebiotics

Ever since Alexander Fleming discovered the antibacterial properties of the fungus *Pencillilum spp.* in 1929 (Fleming, 1929), the world has seen the rapid dominance of antibiotics for the treatment of bacterial infections. However, whilst antibiotics are effective in most cases, they are also inherently associated with a number of drawbacks. When ingested for the treatment of gut infections, for example, antibiotics do not discriminate between the pathogens and the beneficial intestinal microflora (Schrezenmeir and de Vrese, 2001). Furthermore, several gut bacteria are now developing antibiotic resistance, and many of these bacterial infections are recognised as serious health threats (Sanders, 1999).

Global organisations including the World Health Organisation are now suggesting that the use of antibiotics be reduced in human medicine (Avorn *et al.* 2001) and that other means of disease/infection treatment be developed. One such approach that has quickly gained popularity is the concept of probioitcs, a

general term for nutritional supplements containing one or more cultures of living organisms (typically bacteria or yeast) that, when introduced to a human, have a beneficial impact on the host by improving the endogenous microflora (Gibson and Roberfroid, 1995). This is generally achieved by the formation of bacteriocins, competition with pathogens for nutrients as well as lowering of the gut pH by fermentation (Hoerr and Bostwick, 2000; Roberfroid, 2000).

A large amount of research has been undertaken to investigate the probiotic effects of bacteria (reviewed by Hoerr and Bostwick, 2000; Klaenhammer, 2000; Rolfe, 2000; Femia *et al.*, 2002; Gallaher and Khil, 1999; Roberfroid *et al.*, 1998, Roberfroid, 2000; Saikali *et al.*, 2004), showing that improved gut health (including reduced diarrhoea, constipation, inflammatory bowel disease) is often associated with *Lactobacillus* and *Bifidobacterium* spp (Harper *et al.*, 1985; Cherbut *et al.*, 2003; Saikali *et al.*, 2004; Sartor, 2005). Food manufacturers have readily tapped into this new market with the development of probiotic-supplemented foods, fermented dairy foods such as yoghurts appearing to be the preferred choice (Salminen *et al.*, 1998; Roberfroid, 2000).

The use of probiotics can also pose a number of practical difficulties though. First, all foodstuffs are prone to a particular shelf-life, and the effectiveness of the probiotic bacteria can be limited due to the development of acidity by fermentation, development of toxins or loss of viability. Secondly, *L. acidophilus* and *Bifidobacterium spp*. are classified as microaerophillic and anaerobic, respectively, and their survival in an oxygen-based environment is limited (Kailasapathy and Rybka, 1997; Shah, 2000; Talwalkar and Kailasapathy, 2004). Yoghurts incorporate a considerable amount of dissolved oxygen during manufacture, and the packaging materials used are generally not oxygen-impermeable. Limited studies have investigated the viability of *lactobacillus* and *bifidobacteria* spp. in yoghurts, and the available data suggests that the effectiveness of commercial probiotcs may be limited despite the fact that oxygen scavengers are often used (Iwana *et al.*, 1993; Rybka and Fleet, 1997; Shah *et al.*, 1995; Micanel *et al.*, 1997; Vinderola and Reinheimer, 1999; Talwalker and Kailasapathy, 2004).

With the difficulties associated with the survival and maintenance of probiotic cultures, a second approach towards improving gut health has evolved involving the use of prebiotcs, these being “food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria (usually bifidobacteria) in the colon” (Schrezenmeir and de Vrese, 2001). In most instances stimulation of bifidobacteria growth

occurs due to the presence of particular carbohydrates, particularly those that are non-digestible by the enzymes of the human digestive system. These carbohydrates can include fructo-oligosaccharides (FOS) and inulin (Hidaka *et al.*, 1986; Wang and Gibson, 1993; Gibson *et al.*, 1995; Bouhnik *et al.*, 1997; Menne *et al.*, 2000; Tuohy *et al.*, 2001; Kolida *et al.*, 2002; Roller *et al.*, 2004; Euler *et al.*, 2005), transgalactosylated oligosaccharides (TOS) (Tanaka *et al.*, 1983; Ito *et al.*, 1993; Rowland and Tanaka, 1993; Bouhnik *et al.*, 1997; Sazawal *et al.*, 2004) and soybean oligosaccharides (Hayakawa *et al.*, 1990; Saito *et al.*, 1992).

Besides the stimulation of growth of lactobacilli and bifidobacteria, prebiotics have been demonstrated to have several other beneficial gastrointestinal benefits. These can include increased faecal biomass, a mild laxative effect and an osmotic effect in the small intestine (Gibson *et al.*, 1995; Cummings and MacFarlane, 2002; Marteau and Boutron-Ruault, 2002). In addition, other improvements have been reported with prebiotics, including increased immune function (Kolida *et al.*, 2002), reduced dental caries (Cummings and MacFarlane, 2002), stimulation of apototsis (Cummings and MacFarlane, 2002) and an anticarcinogenic effect (Boutron *et al.*, 1996; Taper *et al.*, 1997; Taper and Roberfroid, 1999, 2000, 2002; Wollowski *et al.*, 2001; Cummings and MacFarlane, 2002).

Importantly it has also been shown that intake of prebiotics may help improve calcium uptake (Abrams *et al*., 2005; Cashman, 2006), this being a significant finding as many individuals consume less than two thirds of the recommended daily intake of calcium (Heseker *et al*., 1992; van Dokkum, 1995; see Section 7.1 for further details on calcium intake and problems related to calcium deficiencies). The improvements in calcium uptake with prebiotics are thought to occur in a number of ways. These include the binding of calcium to the non-digestible sugars, thereby preventing the formation of the insoluble hydroxyapatite, and fermentation of the non-digestible sugars in the lower gut to produce acidic conditions, again preventing hydroxyapatite formation (Younes *et al*., 2001). As well, an osmotic effect resulting from prebiotic intake can lead to the opening of gap junctions between mucosal cells in the intestine, thereby increasing calcium absorption through a paracellular route (Abrams *et al*., 2005). Further information relating to the possible improvements in calcium uptake with prebiotics (including honey) is given in Chapters 7 and 8.

1.6.2 Prebiotic Effect of Honey

Recently, a small number of researchers have demonstrated that honey can stimulate the growth of bifidobacteria *in vitro* (Chick *et al.*, 2001; Ustunol and Gandhi, 2001; Kajiwara *et al.*, 2002; Sanz *et al.*, 2005). In a large study funded by the National US Honey Board, 3–5% (w/v) honey significantly enhanced the growth and survival of bifidobacteria BF-1, BF-6 and *B. longum in vitro* when added to inoculated samples of 12% reconstituted non-fat dry milk but no effect on bifidobacteria growth was observed with the equivalent concentrations of either fructose, glucose or sucrose (Ustunol and Gandhi, 2001). Similarly, 5% honey stimulated the growth of the human intestinal bifidobacterium spp., *B. longum, B. adolescentis, B. breve, B. bifidum and B infantis* when cultured *in vitro* in reinforced clostridial medium (Kajiwara *et al.*, 2002). The mean doubling times of these bacteria decreased from 147–690 hours to 9.9–14.3 hours after 48 hours of anaerobic incubation (Kajiwara *et al.*, 2002). In both these studies the effects of honey were equal to or greater than that observed with FOS, gluco-oligosaccharides (GOS) and inulin (Ustunol and Gandhi, 2001; Kajiwara *et al.*, 2002). However, in both of these studies, no control tests were performed to determine what level of bacterial growth would occur due to the glucose and fructose present in the honey. Bergey's Manual of Systematic Bacteriology (1984) states that bifidobacteria will grow on a medium of glucose and/or fructose, thus it is possible that the bacterial growth reported by Ustunol and Gandhi (2001) and Kajiwara *et al.* (2002) was due to the sugar components of the honey. It has been demonstrated in other research, though, that honey can stimulate the *rate of growth* of bifidobacteria when grown *in vitro* on a glucose medium (P. Molan, personal communication). This may be due to the laxative effect of honey (Ladas *et al.*, 1995, Ladas and Raptis, 1999) such that sugars that are normally absorbed in the upper gut are available as an energy source for bacteria in the lower gut.

In line with other prebiotic research, it has also been suggested that the prebiotic effect of honey is due to its oligosaccharide content. As mentioned in Section 1.2.2, honey consists primarily of the monosaccharides glucose and fructose. However, a detailed examination of 27 Spanish honeys revealed that honey can also contain 10–25% minor carbohydrates, including disaccharides and tri-saccharides (Sanz *et al.*, 2004). Astwood *et al.* (1998) has demonstrated the presence of four tetra-saccharides, one penta-saccharide and one hexa-saccharide in New Zealand honeydew honeys, and it is highly likely that there are also other as yet unknown oligosaccharides present in honey.

To conclusively demonstrate that the prebiotic effects of honey are due to oligosaccharides, a group of British and Spanish researchers recently investigated the growth of faecal bifidobacteria and lactobacilli when in the presence of monosaccharide-devoid honey in an *in vitro* fermentation system (Sanz *et al.*, 2005). After removing the glucose and fructose from a honeydew sample via nanofiltration, yeast treatment and adsorption onto active charcoal, the remaining oligosaccharide mixture was shown to increase the bacterial populations after 12 hours of fermentation, with a prebiotic index (PI) in the range of 3.38–4.24 (the PI is calculated to give a comparable index between the growth of beneficial faecal bacteria (*e.g.* bifidobacteria, lactobacilli and eubacteria) and the less desirable ones (*e.g.* clostridia and bacteroides)) related to changes in the total number of bacteria (Palframan *et al.*, 2003)). Unlike previous studies that reported that honey appeared to have a prebiotic effect that was equal to or greater than that of FOS, this study demonstrated that the prebiotic potential of neither the total honey nor the oligosaccharide mixture was as potent as the FOS (PI = 6.89; GOS and inulin were not investigated in this study). However, this same study did demonstrate that lactic acid and acetic acid production was at least as high with honey and honey oligosaccharides as with FOS, suggesting that growth of bifidobacteria was enhanced to an equal extent in these samples.

Similarly, Shamala *et al* (2000) showed that *Lactobacillus plantarum* counts increased 10–100 fold when grown *in vitro* in the presence of 1% honey compared with 1% sucrose or 1% lactose + 1% glucose. In contrast, Curda and Plockova (1995) demonstrated that the growth of *Lactococcus lactis* bacteria in skim milk was inhibited by the addition of 5–10% honey. The effects of honey on the growth of lactic acid bacteria therefore require further investigation.

With the exception of the two studies mentioned earlier (Astwood *et al.*, 1998; Sanz *et al.*, 2004), there appears to be very little information available as to the oligosaccharide content of honey. A group of German researchers have identified several oligosaccharides (1-kestose, neokestose, nystose, 6-kestose, raffinose, stachyose, isomelezitose and fructocsylisomelezitose) in honey, and have demonstrated that these all appear to be resistant to digestive enzymes (Rittig, 2001). To date, only two *in vivo* studies have investigated the prebiotic effect of honey (Shamala *et al.*, 2000; Ezz El-Arab *et al.*, 2006). In the earlier study, lactic acid bacteria counts were significantly increased in rats fed 2 grams of honey per day compared with those fed 2 grams of sucrose per day after 6 weeks of feeding (7.8 vs 5.8 Log_{10} viable counts; Shamala *et al.*, 2000). Similarly, the addition of 10% cotton honey to a powdered AIN-93 diet significantly

increased counts of bifidobacteria and lactobacilli by a mean of 2.3 and 1.07 Log_{10} colony forming units / gram caecum content, respectively, in Swiss Albino mice after 2 months compared with control animals fed the powdered di*et al*one (Ezz El-Arab *et al.*, 2006).

1.7 OTHER HEALTH PROPERTIES OF INGESTED HONEY

In addition to the well-recognised bioactive properties of honey that have been discussed earlier in this chapter (*i.e.* antibacterial, antioxidant and prebiotic effects), recent research has also discovered a number of other properties that may support health and wellness.

1.7.1 Gastric Health

In recent years, a small number of studies have been undertaken to assess the gastro-protective effects of honey in rats (Ali *et al.*, 1997; Ali and Al-Swayeh, 1997; Gharzouli *et al.*, 1999; 2002; Mahgoub *et al.*, 2002; Ali, 2003). In the earlier study of Ali *et al.* (1997), Wistar rats given oral solutions of honey (0.078–0.625 g/kg body weight) 30 minutes prior to ischaemia- and reperfusion-induced gastric lesions exhibited significantly fewer gastric lesions, and reduced intraluminal bleeding and vascular permeability compared with animals pre-treated with water only. Similar results were also obtained with pre-treatment with the free-radical scavenger dimethyl sulphoxide (DMSO) (0.02–0.08 g/kg body weight, intraperitoneally) (Ali *et al.*, 1997), suggesting that free radical damage may be involved in the pathogenesis of ischaemia-reperfusion-induced mucosal injuries, and that the gastro-protective effects of honey may be due to its antioxidant properties.

Similarly, Ali and Al-Swayek (1997) reported that natural honey (0.078–0.625 g/kg body weight) prevented the increase in vascular permeability that was observed following exposure to ethanol, although this effect was diminished if the animals were pre-treated with the sulphydryl blocker *N*-ethylmaleimide (0.05 g/kg) prior to being administered the honey. Thus, it has been suggested that the protective effects of honey may be mediated through sulphydryl-sensitive processes, as well its antioxidant content. Similar conclusions were also drawn by Ali (2003) when it was shown that both honey (0.312–1.25 g/kg body weight) and sulcralfate (0.25–1.0 g/kg) prevented the formation of ammonia-induced gastric lesions, and reversed the decrease in non-protein sulphydryls that resulted from the ammonia treatment.

In two studies undertaken in Algeria (Gharzouli *et al.*, 1999; 2002) honey has again been demonstrated to have gastro-protective effects, it preventing the gastric lesions induced by ethanol (Gharzouli *et al.*, 1999; 2002) and indomethacin and acidified aspirin (Gharzouli *et al.*, 2002). Importantly, both of these studies also tested the effects of a mixture of glucose, fructose, sucrose and maltose, and demonstrated gastro-protective effects that were only slightly less than those of the honey, suggesting that the protective effects of the honey may be substantially due to its carbohydrate content. In the two studies by Gharzouli *et al.*, however, the concentrations of the four sugars used in the sugar mixture were supposed to be representative of the levels found in the honey (38.2% fructose, 31.3% glucose, 1.5% sucrose and 7.3% maltose). But, due to the authors not taking the density of honey into account, and adding only 17.5 ml of water rather than making the solution to a total of 100 ml, the sugar mixture they prepared was considerably more concentrated than that found in a typical honey. It has suggested, therefore, that the gastro-protective effects of the sugar mixture were most likely due to it simply forming a physical barrier over the mucosal surface of the stomach (Ali, 2001).

Honey may also have a possible role in the improvement of gastric health, via the improvement of naturally occurring peptic ulcers. In a small number of *in vitro* studies, Manuka honey has been shown to inhibit the growth of *Helicobacter pylori* (Al Somai *et al.*, 1994; Osato *et al.*, 1999), the organism thought to be responsible for the development of dyspepsia and peptic ulcers. In the earlier study (Al Somai *et al.*, 1994), Manuka honey was shown to have bacteriostatic properties against *H. pylori* at concentrations of 5% (v/v), with most clinical strains showing complete inhibition of growth with honey concentrations of 20% (v/v). Importantly though, solutions of glucose, fructose and their combinations have also been shown to inhibit the growth of *H. pylori* at concentrations ≥ 15% (Osato *et al.*, 1999) suggesting that the inhibition may be due to the osmotic action of the honey/sugar solutions rather than any inherent antibacterial activity. In addition, whereas Manuka honey has been shown to be effective at retarding the growth of *H. pylori in vitro*, it has not been demonstrated to have any effect *in vivo*. In a small clinical study, McGovern *et al.*, (1999) reported that all patients (n=6) remained positive for *H. pylori* four weeks after a 2-week regime of a tablespoon of Manuka honey, taken four times a day. No other clinical studies appear to have investigated the efficacy of honey as a treatment for dyspepsia or gastric ulcers, although it would certainly be of interest to further study its clinical efficacy given the above findings that honey can minimise the formation of gastric

ulcers when taken as a preventative measure. In particular, ammonia is liberated in the stomach of *H. pylori*-infected individuals, and it has been implicated in the pathogenesis of peptic ulcer diseases (Ali, 2003). Thus, honey may offer an effective therapeutic treatment for gastric ulcers, both via its antibacterial effect on the *H. pylori* organisms directly, and via prevention of ammonia-induced gastric lesions.

Honey may also be an effective treatment for ulcerative colitis of the gut, as limited research in rats has demonstrated that honey significantly reduced intestinal mucosal injury scores when given as a 50% solution (2 ml) rectally for 7 days following induction of colitis (Bilsel *et al.*, 2002). Importantly, in this same study, treatment with prednisolone and disulfiram (pharmaceuticals that are standard therapy for inflammatory bowel disease) had no effect, the degree of intestinal damage (ulcers and erosions) being similar between these animals given these two treatments and those in the control saline group. Similarly, in a second animal study (Mahgoub *et al.*, 2002), honey provided dose-dependent protection against acetic acid-induced colonic damage, when taken orally and rectally for four days, with induction of colitis on day three (*i.e.* after the first two days of honey treatment). Honey at a concentration of 5 g/kg body weight afforded nearly 100% protection, whilst a mixture of glucose, fructose, sucrose and maltose (as in honey) had no protective effect (Mahgoub *et al.*, 2002). In both studies, honey also restored malondialdehyde levels (an indicator of lipid peroxidation) back towards normal levels (Bilsel *et al.*, 2002; Mahgoub *et al.*, 2002).

In other clinical studies, it has also been suggested that honey may aid in the treatment of diarrhoea and gastroenteritis (Chatterjee *et al.*, 1978; Finberg, 1980; Salem, 1981; Haffejee and Moosa, 1985; Linnett, 1996). In one well-designed study (Haffejee and Moosa, 1985), 169 infants presenting with gastroenteritis were given either standard rehydration therapy (oral and/or intravenous fluids containing glucose and electrolytes) or honey-based rehydration therapy (oral and/or intravenous fluids, with the oral solution containing honey in place of glucose such that the electrolyte content was the same). When taken as a whole, there were no significant differences between the two treatments for the mean recovery time, nor the mean rehydration time; however, when only those patients with bacterial gastroenteritis were considered, infants given honey (50 ml/l) had significantly reduced mean recovery times compared with the control group (58 *vs* 93 hours). It has been suggested that honey may have this effect due to its high sugar content promoting absorption of

sodium and water from the gut (Nalin, 1975), although it is likely that other factors may be involved also. In contrast, honey has also been suggested as a good remedy for constipation – this being due to the fact that consuming honey has been shown to have a laxative effect *in vivo* (Ladas *et al.*, 1995, Ladas and Raptis, 1999).

1.7.2 Anti-Cancer Effects of Honey

A small number of studies (Swellam *et al.*, 2003; Orsolic and Basic, 2004; Orsolic *et al.*, 2005) have investigated the anti-cancer properties of honey in recent years, with interesting results. In the earlier study (Swellam *et al.*, 2003), honey (1–25%) significantly inhibited the growth of bladder cancer cell lines when tested *in vitro*. In addition, intralesional injections of honey (6 and 12%) significantly reduced the growth of tumours resulting from cancer cells that were implanted into the abdomen of mice (Swellam *et al.*, 2003). No comparisons with other sugar solutions were undertaken in this study, but it was suggested that the effects may have been due to its chemical constituents as another study has reported that administration of hydroxycinnamates from honey significantly inhibited benzopyrene-induced neoplasia of the forestomach in mice (Wattenburg et al., 1980).

Similarly, solutions of honey (1–2 g/kg bodyweight) given orally via a gastric tube for 10 days significantly reduced metastasis formation in mice and rats with transplanted carcinoma in the lungs, but only when it was taken as a preventative measure (Orsolic and Basic, 2004; Orsolic *et al.*, 2005). In contrast, animals receiving honey therapy that began 2 days after tumour cell inoculation had no reduction in tumour nodule formation, with a few animals even exhibiting enhanced tumour growth in their lungs (Orsolic and Basic, 2004). Importantly, no controls were carried out with these studies, although it was also reported in one study that royal jelly also reduced the formation of metastases when it was co-administered with the tumour cells (Orsolic *et al.*, 2005).

1.7.3 Effects on Biochemical and Physiological Indices

The contents of this section form the basis of much of the work presented in this thesis, therefore they are discussed in detail in the following chapters. For this reason, this section will provide a brief overview only.

Honey has been shown to have a number of effects on biochemical indices, although most of this data has come from only a small number of studies (Al-Waili, 2003a, 2004b). In a small clinical study (n=24), inhalation of an aerosol

of honey (60% w/v) for 10 minutes resulted in a lowered blood glucose level and elevation of plasma insulin and C-peptide levels (Al-Waili, 2003a). In contrast, inhalation of 10% dextrose led to a mild reduction in plasma insulin levels and no change in blood glucose (Al-Waili, 2003a). In addition, blood pressure was mildly reduced, and there was an 11–16% increase in peaked expiratory flow rate in patients given honey, but no significant changes were observed for either endpoints in patients given distilled water or dextrose (Al-Waili, 2003a). Similarly, in a second small noncomparative clinical study (n=8) oral consumption of honey (75 g in 250 ml water) once-daily for 15 days resulted in a reduction in total cholesterol (7%), LDL-cholesterol (1%), triglycerides (2%) and C-reactive protein (7%) compared with baseline levels, although these did not reach statistical significance (Al-Waili, 2004b). As well, the same quantity of honey (once-daily for 15 days) decreased total cholesterol (8%), LDL-cholesterol (11%), and C-reactive protein (57%) in patients (n=5) who had elevated cholesterol (> 200 mg/dl) prior to beginning the study, the changes in total cholesterol and C-reactive protein both reaching statistical significance ($p < 0.05$) (Al-Waili, 2004b).

Intravenous administration of honey (25 g in 500 ml saline) in healthy sheep was also shown to improve renal and hepatic function, bone marrow function, and the fasting lipid profile compared with baseline levels (Al-Waili, 2003b) However, in most cases, the levels reported with honey were not significantly different from those reported for control sheep given dextrose. In the same study, honey (80 g in 500 ml water), given four times in 17 days, numerically reduced serum glutamic oxaloacetic transaminase levels, triglyceride and total cholesterol levels, and significantly elevated serum protein, haemoglobin and white blood cell counts compared with baseline levels (Al-Waili, 2003b). Reductions in triglyceride levels were significantly lower in honey-treated sheep compared with those given dextrose at 20 days (17.3 vs. 21.7 mg/dl; $p = 0.02$) but no other differences between the two treatment groups were observed at 10 or 20 days. In addition, in a small non-comparative study, honey (80g in 250 ml water) increased serum phosphate levels, and decreased serum creatinine and blood urea nitrogen levels after 10 hours in another study (Al-Waili, 2005). Furthermore, in a small clinical (n=10) study daily consumption of honey (1.2 g/kg bodyweight in 250 ml water) for 2 weeks decreased creatinine kinase (33%), lactic acid dehydrogenase (41%), plasma ferritin (11%), aspartate transaminase (22%) and alanine transaminase (18%) levels (Al-Waili, 2003c). Again, this study was non-controlled and non-comparative, and it is not known

what effects were simply time related, nor whether these responses were due to other components of the diet.

It has also been shown that honey can have differential effects on blood sugar levels compared with other sugars (Shambaugh *et al.*, 1990). Honey has a lower glycemic response than sucrose (Shambaugh *et al.*, 1990), and plasma glucose levels have been shown to be lower in both healthy and diabetic patients (Samanta *et al.*, 1985; Al-Waili, 2004b) as well as in sheep (Al-Waili, 2003b) and rabbits (Akhtar and Khan, 1989) who have consumed honey compared with those that have consumed dextrose, sucrose or fructose. In one recent study comparing honeys of differing floral sources, GI values were shown to not differ significantly between samples, and no relationship between GI and the fructose to glucose ratio was observed (Ischayek and Kern, 2006). These authors reported that the mean GI values of US honeys to range from 69.2–74.1 (Ischayek and Kern, 2006), although Arcot and Brandmiller (2005) reported the GI values of Australian honeys to be lower (range 35–72).

Limited research has also suggested that consumption of honey can have an impact on the levels of various minerals in the body (Knott *et al.*, 1941; Al-Waili, 2003c, Ariefdjohan *et al.*, 2005). In the study by Al-Waili (2003c), daily intake of honey (1.2 g/kg bodyweight) for two weeks increased vitamin C (47%), uric acid (12%), serum iron (20%) and serum copper (33%) levels compared with baseline levels. In the other two studies, honey was shown to improve calcium uptake in humans (Knott *et al.*, 1941) and in rats (Ariefdjohan *et al.*, 2005). In the earlier study, calcium retention was shown to be higher in infants fed formula containing honey compared with those fed formula alone (Knott *et al.*, 1941), whilst in the second study, a single oral gavage of honey (500 or 800 mg) plus calcium led to increased absorption of radioactively-labelled calcium (this study is published as an abstract only). It has been hypothesised that this action was due to the prebiotic effect of honey (discussed in Section 1.6.2).

1.7.4 Effects on Immunity

Honey has been demonstrated to have an effect on various aspects of immunity, and anecdotal evidence suggests that it can stimulate immune function. In *in vitro* studies, honey (at concentrations of 0.1 or 0.2%) has been shown to stimulate the proliferation of B- and T-lymphocytes (Abuharfeil *et al.*, 1999) and to stimulate antibody production during primary and secondary immune responses (Al Wali and Haq, 2004). As well, limited research has shown that honey can stimulate the release of tumour necrosis factor α in unprimed

monocyte cells (Tonks *et al.*, 2001, 2003). The factors in honey responsible for this are currently unknown; however, it has been demonstrated that the same responses are not seen with glucose solutions diluted to the same extent (Abuharfeil *et al.*, 1999) or with mixed sugar solutions made up to represent honey (Tonks *et al.*, 2003).

Research has demonstrated that sugars that are slowly absorbed can lead to the formation of short chain fatty acid (SCFA) fermentation products in the gut (Kruse, 1999). SCFA are commonly formed after ingestion of prebiotic foods, and with the prebiotic effects of honey established *in vitro* (refer to Section 1.6.2) it is possible that the ingestion of honey may also result in their formation. A number of studies have demonstrated, either directly or indirectly, that SCFA can have immunomodulatory properties (reviewed by Schley and Field, 2002). Thus it is possible that honey may stimulate the immune system via the presence of fermentable sugars in the gut

It is also possible that non-sugar components of the honey may be responsible for its immuno-modulatory effects. Tonks et al. (2007), for example, have recently reported that a 5.8 kDa component of Manuka honey is capable of stimulating immune function *in vitro*. These authors determined that the compound was not a lipopolysaccharide, amino acid, vitamin or mineral, but further investigations are required to elucidate the nature of the compound. It is also possible that the antioxidant content of the honey may contribute to its immuno-modulatory effects. Although there are no studies directly investigating the effects of honey antioxidants on immune function, other antioxidant compounds have been shown to stimulate immune function *in vitro* (Sabongi *et al.* 1997) and *in vivo* (De La Fuente *et al.* 2002).

Furthermore, honey is capable of providing a ready supply of glucose to macrophage cells, thereby enabling the “respiratory burst” that produces hydrogen peroxide (hydrogen peroxide is the main substance used by these cells to destroy bacterial activity) (Ryan and Majno, 1977). The acidity of honey also aids in the bacteria-destroying action of macrophages, as an acid pH inside the phagocytic vacuole is involved in killing ingested bacteria (Ryan and Majno, 1977).

1.8 OVERVIEW OF THIS THESIS

The aim of this thesis was primarily to investigate the health benefits of consuming honey, as compared with sucrose, within the range of total sugar concentrations that are in the typical New Zealand diet. It hypothesised that

honey may lead to better weight gain, calcium uptake and cognitive performance compared with sucrose during either short- or long-term feeding (see Section 1.8.1 for the specific aims of the different studies).. The majority of this work was achieved using the rat as a model, although a number of small *in vitro* studies were also undertaken. As the majority of the research in this thesis was funded by Fonterra Brands, New Zealand Ltd, the research was carried out with the honey and the sucrose incorporated into diets that were formulated using milk products.

This thesis presents a small amount of data from *in vitro* analyses that were designed to investigate whether the bioactivities of honey would be altered in the presence of milk (see Chapter 3), and six animal trials to assess various endpoints (see below). Three of the studies (Chapters 4, 5 and 7) were carried out at Crop and Food Research (CFR) in Palmerston North prior to the conception of this thesis; however, they are included in this thesis thanks to the generosity of management at Crop and Food Research. The additional three studies (Chapters 6, 8, 9 and 10) were fully funded by Fonterra Brands Ltd. These trials were designed to re-evaluate the earlier work carried out at Crop and Food Research, and to further investigate other parameters.

1.8.1 Aims of This Thesis

The aims of the 6 animal studies were as follow:

Study 1: To determine whether honey, sucrose and mixed sugars as in honey would differ in their effects on protein uptake and protein metabolism in weanling rats.

Study 2: To ascertain whether honey, sucrose and mixed sugars as in honey would differ in their effects on weight gain in adult rats.

Study 3: To determine whether honey, sucrose, mixed sugars as in honey and a sugar-free diet would differ in their effects on weight gain in young rats.

Study 4: To ascertain whether honey and sucrose would differ in their effects on calcium uptake in calcium-deficient rats.

Study 5: To determine whether honey and sucrose would differ in their effects on calcium uptake in rats that were not calcium deficient.

Study 6: To ascertain whether honey, sucrose and a sugar-free diet would differ in their effects on cognitive function and age-related parameters (weight gain, immune function, cholesterol profile, bone strength) after long-term feeding in rats.

In both the *in vitro* studies and the animal trials, commercially available honeys were chosen. Honeys from specific floral sources that have known bioactivities but may not be readily accessible to either the general public or food manufacturers (*i.e.* Manuka honeys) were avoided, as these are not representative of the majority of honeys available on the world market. In the various studies, honeys were selected based on their levels of hydrogen peroxide-producing activity, antioxidant content or on the composition of the various sugars (*i.e.* high fructose content); however, in all cases the honeys were still representative of those that are widely available for purchase.

Chapter Two:

Methods and Materials

The majority of methods used in this thesis are given in the relevant chapters, specifically why the particular study and diet designs were chosen., and the reader is directed to the individual chapters for more information. However, methods and analyses that were routinely used are given below.

2.1 MATERIALS

2.1.1 Trial Locations

The work presented in this thesis consists of three animal trials (Chapters 4, 5 and 7) that were carried out at Crop and Food Research in Palmerston North and three trials that were undertaken at Waikato University, Hamilton. All *in vitro* studies were carried out at Waikato University.

Trials undertaken at Crop and Food Research were carried out at the Food Evaluation Unit (FEU) in a room maintained at a temperature of 22±1°C, humidity of 60 (±5)%, air exchange of 12 times/hour, and with a 12 hour light/dark cycle. Trials carried out at Waikato University were in either the rodent room in the Animal house (Building BL5) or in rooms in the Animal Behaviour Research

Facility (Building BL10). Both buildings were maintained at 22±1°C with a 12 hour light/dark cycle.

Due to the age of the Animal House (BL5), humidity and air exchange were not mechanically controlled. In this rodent room, humidity was assessed twice weekly using a humidistat, and maintained at 60 (±10)% by adding a dish of water to the room as required. Fresh air was constantly circulating throughout the room due to the presence of an external fan on the exterior wall. A second fan, on an interior wall, moved air through the room.

Unless specified otherwise, animals were housed in standard rat cages that had plastic bottoms and metal grid tops. The final measurements of each cage was 45 x 25 x 30 cm high.

2.1.2 Experimental Animals

Weaned, Sprague Dawley male rats (of various ages as detailed in the specific chapters) were used in all the feeding studies described in this thesis. Animals were bred at, and supplied by, the FEU, Crop & Food Research, Palmerston North (for trials carried out at Crop and Food Research), or by AgResearch, Ruakura Campus, Hamilton (for trials carried out at Waikato University).

2.1.3 Experimental Diets

All diets were prepared by Crop and Food Research in Palmerston North. Diets were prepared by mixing the dry ingredients in large commercial mixers (20 kg capacity) for a minimum of five minutes. The liquid ingredients (water and oil) were then added by pouring slowly into the mixture while the mixer was turning on low speed. Lastly, the honey (warmed to room temperature one hour prior to mixing) was added to the mixture in a similar manner. All honeys used were fairly liquid in nature, and no difficulties with the diet formulations were encountered. Water was added to all non-honey diets to ensure that all diets had the same moisture content (to account for the fact that honey contains on average 18–20% water (Molan 1996); see specific chapters for actual amounts). All diets retained a similar powdery consistency after mixing, and no stickiness or clumping was observed with the honey diets. Percentage dry matters were determined for all diets in all trials. This was performed by placing 0.5 ± 0.1 g into weighed 20 ml beakers (three triplicates per sample) and drying for 16 hours at 105^{o}C. Combined weights of the sample plus beaker were determined both before and after drying and the % dry matter calculated.

Diets were prepared no more than 10 days prior to commencing the study and were kept in the dark at 4°C or -18°C for the duration of the study. Unless specified otherwise, diet ingredients were sourced from the following places:

Amylose	HA1, Penford Australia Ltd
Cellulose	Avicel PH102, Commercial Minerals Ltd, Auckland, NZ
Corn Oil	Davis Trading Company, Palmerston North, NZ
Egg Albumin	Zeagold Products, Auckland, NZ
Fructose	F0127, Sigma Chemical Company, Australia
Glucose	G8270, Sigma Chemical Company, Australia
Lactic casein	80 mesh, New Zealand Milk Products
Lactalbumin,	Alatal 825, New Zealand Milk Products
Maltodextrin	Davis Trading Company, Palmerston North, NZ
Maltose	M5885 , Sigma Chemical Company, Australia
Starch	Davis Trading Company, Palmerston North, NZ
Sucrose	S7903, Sigma Chemical Company

'Mixed sugars' were prepared based on a sugar analysis profile of the honey used in Chapter 5 & 6 (Agriquality, Auckland NZ) and formulated to contain equivalent amounts of glucose, fructose, sucrose and maltose. Maltodextrin was used to replace the various minor oligosaccharides present in honey.

Standard rodent vitamin and mineral mixes were prepared to comply with the requirements for growth as specified by the National Research Council (1995). In general, a sugar-free vitamin mix was prepared by mixing 39.57 g of vitamins with 60.43 g cellulose, and adding to the rodent diets at a level of 5 g/kg. This mixture supplied (per kg of diet): 5.0 mg retinol acetate, 200.0 mg DL-α-tocopherol acetate, 3.0 mg menadione, 5.0 mg thiamin hydrochloride, 7.0 mg riboflavin, 8.0 mg pyridoxine hydrochloride, 20.0 mg D-panothenic acid, 2.0 mg folic acid, 20.0 mg nicotinic acid, 1.0 mg D-biotin, 200.0 mg myo-inositol, 1500 mg choline chloride, 25.0 μg ergocalciferol, μg 50.0 cyanocobalamin. The mineral mix was prepared and added to all diets at a level of 50 g/kg to supply (per kg of diet): 6.29 g Ca, 7.79g Cl, 1.06 g Mg, 4.86 g P, 5.24 g K, 1.97 g Na, 1.97 mg Cr, 10.7 mg Cu, 424 mg Fe, 78.0 mg Mn, 48.2 mg Zn, 29.0 μg Co, 152 μg Mo, 151 μg Se. A calcium-free mineral mix was prepared for use in Chapter 7 by replacing

the calcium phosphate in the diet with cellulose and adding the same molar quantity of phosphate.

For all trials, honeys were sourced from supplies at the Honey Research Unit, Waikato University or from apiarists in New Zealand. Different honeys were required for the different trials, and details are provided in the relevant chapters

In all the studies undertaken, the rats seemed to have no issues with eating the different diets. It has been suggested that rats do not like a predominantly starch-based diet as it can get it their nose and whiskers, so care was always taken to ensure that either the fat content was >10% or that sugars of some sort were added to the diet. The diets were therefore in accordance with recommendations for rodents as detailed in the AIN 93 diet.

2.1.4 Ethical Approval

All studies was carried out with ethical approval from the Palmerston North Crown Research Institutes' Animal Ethics Committee (if carried out at Crop and Food Research) and from the Waikato University Ethics Committee (for studies carried out at Waikato University).

2.2 METHODS

Unless specified otherwise, samples of diet and blood were sent to external sources (Hospital Laboratory, Waikato Hospital, Hamilton and the Nutrition Laboratory, Institute for Food, Nutrition and Human Health, Massey University, Palmerston North) for measurement of the various endpoints. Both laboratories employed were IANZ accredited facilities, and standard methodologies were used. Where the analyses are published methods they are not given in detail in this Section. Readers are directed to the appropriate references if further information is required. AOAC numbers refer to the methods reported in the Official Methods of Analysis of AOAC International (1995).

In all instances, whole blood and/or serum samples were prepared at the university within 2 hours of drawing the blood, and taken to the laboratory for analysis within 8 hours. Details for sampling procedures, and sample preparation are given in the relevant chapters.

2.2.1 Gross Energy

Gross Energy (kJ/g) of diet samples were determined using Bomb calorimetry following the methodology in the AC-350 LECO Instrument Manual (see Appendix 1). In brief, the diet samples were pelletised using compaction,

and 0.5-1.0 g loaded automatically into the AC-350 Automatic Calorimeter. Net caloric values were obtained by the machine using the following:

$$Q_{net} = Q_{gr} - 10.30(H_{ar} \times 9)[BTU \bullet lb^{-1}] = Q_{gr} - (HF \times H_{ar})\ [BTU \bullet lb^{-1}]$$

Where: Q_{net} = the net calorific value in BTU/lb
Q_{gr} = the gross caloric value in BTU/lb
10.30 = the latent heat of vaporisation of water in BTU/lb
H_{ar} = the known weight percent of hydrogen as-received basis in the sample
9 = the approximation for the ratio of the molecular weight of water and diatomic hydrogen
BTU = British thermal units
lb = pound

2.2.2 Calcium Measurements

Measurement of dietary calcium content was determined by preparation method AOAC 968.08D followed by colorimetric analysis using the cresol phenolphthalein complexone (CPC) method. For method AOAC 968.08D, samples of diet (2–10 g) were dry-ashed by heating in a furnace at 550^{o}C for four hours. The samples were then cooled, and 10 ml of 3*N* HCl added to each, before boiling for 10 minutes. The samples were then cooled, filtered into 100 ml volumetric flasks, and diluted to volume with distilled water. Subsequent dilutions with 0.1–0.5*N* HCl were used to bring the sample solutions into analytical range.

Bone calcium (after HCl digestion) and serum calcium content were also determined using the CPC method. For this method, 0.1 ml samples were incubated with 2 ml of the cresol colour reagent (25 mg *o*-cresolphthalein complexone powder, 250 mg of 8 hydroxy-quinoline and 15 ml concentrated HCl in a total volume of 250 ml with distilled water) and 2 ml of 2-amino, 2-methyl, 1-propanol (AMP) buffer (37.8 ml of AMP reagent in 150 ml of distilled water, adjusted to pH to 10.7 with 6*N* HCl and made up to 250 ml with distilled water) at room temperature for 15 minutes. The optical density of the test samples were then measured in a spectrophotometer at 540 nm and compared to working standards (5, 7.5, 10, and 12.5 mg/100 ml calcium).

2.2.3 Fat and Protein Measurements

Total body fat content was measured using Soxhlet fat extraction as detailed by Firth *et al*. (1985) and Hawthorne *et al*. (2000). In brief, samples of ground tissue (1.0–3.5 g) were placed in porous clay thimbles in a soxhlet extractor, held in place with glass wool. The samples were then extracted with diethyl ether over a period of approximately 40 hours.

Tissue protein content was determined using total combustion method AOAC 968.06 using a Coleman Model 29A Nitrogen Analyser. In brief, upper and lower furnace temperatures were set to 850–900^{o}C. Samples were ground (such that there were able to pass through a No. 30 sieve), and placed into weighed 'combustion boats'. The weight of the combined boat plus sample was then recorded. The combustion boats were loaded into the combustion tubes and volumes of Co_3O_4 and CuO powders (equal to the sample volume) added. The prepared combustion tube was then installed in the N_2 analyser. KOH (45%) was added up to the calibrating mark of the nitometer. The combustion cycle control was set to START and allowed to proceed through its cycle. The amount of nitrogen/protein present was determined automatically by the Nitogen Analyser using calculations as detailed in the AOAC methods of analysis (1995).

2.2.4 Glycated hemoglobin (HbA1c)

Glycated hemoglobin levels were measured using an automated HPLC with a boronate phenylboronic acid affinity column (Primus Diagnostics, Cat. # 3-02-0078) and colorimetric detection. Briefly, for each sample, 5 ml of blood was diluted/hemolysed in 1 ml HPLC grade water (pH 9.0) and injected automatically into the HPLC equipped with the affinity column (the glycated component of the sample is retained on the column due to its diol groups complexing with the boronate). The glycated hemoglobin was eluted using Tris buffer containing EDTA, sorbitol and acetonitrile (pH 8.0, supplied by Primus Diagnostics; molarity not reported). Absorbance of the bound and unbound fractions, measured at 415 nm (using a UV detector) was used to calculate the percentage of glycated hemoglobin. Calibrated samples (Primus Diagnostics, Cat. # 04-01-0016) were run as appropriate controls.

2.2.5 Fasting Lipid Profiles

Fasting lipid profiles were performed by following standard Roche methods, followed by colorimetric detection with a P800 Hitachi. Total cholesterol was measured as described by Trinder (1969) using Roche Diagnostic reagents

(Cat. # 11491458216). A reference range of 3.0–5.0 mmol/l cholesterol was used, with a lower detection limit of 0.1 mmol/l.

HDL-cholesterol was determined by the Roche HDL-C Plus homogenous method (Friedwald *et al*., 1972) using Roche Diagnostic reagents (Cat. # 04713214109) to exclude non-HDL cholesterol and triglycerides. The method has been shown to be linear up to 3.12 mmol/l, with a lower detection limit of 0.08 mmol/l.

Triglyceride levels were determined following the method of Trinder (1969) using Roche Diagnostic reagents (Cat. # 11730711216). The assay has a reference range of 0.3–1.9 mmol/L, with a lower detection limit of 0.05 mmol/l.

LDL-cholesterol levels were determined by calculation using the following:

[LDL-C] = ([triglycerides] / 2.2) – [HDL]

Chapter Three:

In Vitro Honey-Milk Interaction Studies

As the majority of this research was funded by Fonterra Brands Ltd, the primary aim of many of the studies in this thesis was to determine the health benefits of honey in a diet that also contained milk products, a series of *in vitro* studies were designed to ascertain whether the combination of milk and honey would affect the various bioactivities of honey. For all experiments both skim milk and whole milk, and three honeys of differing floral sources, were tested.

A review of some of the locally available milk products (flavoured milk drinks, yoghurts *etc*) showed that sugars, other than lactose, are often present in these products, but that the level of added sugar generally does not exceed 6%. It was therefore deemed likely that any addition of honey to a milk-based product would not occur at levels higher than 10%. For this reason, unless reported otherwise, only honey concentrations of 10% and 100% (full strength) were assessed in this chapter.

3.1 ANTIBACTERIAL ACTIVITY

3.1.1 Introduction

The antibacterial activity of honey is well established in the literature (see Section 1.3) and it is often thought to be one the key bioactive properties of

honey. With the exceptions of Manuka honey, the antibacterial activity of honey is primarily due to hydrogen peroxide production by the enzyme glucose oxidase. However, the levels of hydrogen peroxide that may be present *in vivo* after consuming honey can vary substantially. This can depend on floral source and factors directly affecting the glucose oxidase activity (see Section 1.3), as well as factors present after consumption that may affect the stability or breakdown of hydrogen peroxide.

Importantly, it is possible that there could also be interactions occurring between the honey and other food products consumed that could affect the overall levels of antibacterial activity observed. Milk, for example, is also known to contain a number of antibacterial proteins (including lactoperoxidase, lactoferrin and lysozyme; Ekstrand and Bjorck, 1986) although activity due to these is likely to be negligible, at best, after pasteurisation and spray-drying. As well, recent studies have demonstrated that a number of bioactive peptides derived from milk proteins can also exhibit antibacterial activity (Malkoski *et al.*, 2001; Florisa *et al.*, 2003; Campagna *et al.*, 2004). Research suggests that some of these antibacterial peptides are formed in the gut, after ingestion (Campagna *et al.*, 2004), suggesting that they could indeed influence the overall level of antibacterial activity observed.

Furthermore, measurement of antibacterial activity using the method used in this chapter is also a way by which the levels of hydrogen peroxide generated in the honey can be determined. This is particularly significant as it has been suggested in *in vitro* studies that the hydrogen peroxide in honey may have an insulin-mimetic effect (Heffetz *et al.*, 1990), thereby affecting glycemic control. This, in turn, could have implications for blood sugar regulation leading to reduced hyperglycemia, and possibly even improved weight control (discussed in Chapter 6). In addition, because Glucose oxidase produces hydrogen peroxide and gluconic acid in a 1:1 ratio, measurement of hydrogen peroxide activity also gives an indication of the amount of acid produced. It has been suggested that the acidity of honey may help to keep calcium soluble in the gut for longer, thereby increasing calcium absorption: this is investigated in Chapters 7 and 8.

Thus a simple experiment was designed to measure the antibacterial activity of three different honeys either alone, or in combination with pasteurised whole or skim milk. The assay used is based on the work of Allen *et al.,* (1991).

3.1.2 Methods

3.1.2.1 Sample Preparation

Milk samples were prepared from commercially available Anchor brand milk powders, obtained from the local supermarket. Both skim milk and whole milk were prepared following the directions on the packet.

Three honeys that had previously been shown to produce detectable levels of hydrogen peroxide were assayed for antibacterial activity:

- RE7 (rewarewa)
- PS3 (mixed pasture)
- HD19 (honeydew)

Samples of each honey were diluted in distilled water, skim milk and whole milk to a concentration of 25% w/v (the concentration required for assay, as reported by Allen *et al.* (1991); see below) and to 10% w/v (the concentration of honey likely to be used in a milk-based product). The honey samples were diluted immediately prior to assaying, and were mixed well using orange sticks to ensure that the honeys were fully dissolved. Samples of milk (100%) were also assessed for antibacterial activity.

3.1.2.2 Assay of Antibacterial Activity

Antibacterial activity was assessed using a standard agar well diffusion assay as described by Allen *et al.,* (1991). In brief, large square plates (Nunc bioassay dishes 243 x 243 x 18 mm) were prepared by mixing 100 μl of an 18 hour culture of *Staphylococcus aureus* bacteria (corrected to 0.5 absorbance units) in Trypticase Soy Broth (BBL) into 150 ml of sterilised nutrient agar (Difco) after it had cooled to 50^{o}C. The plates were poured on a level surface immediately after mixing, and were stored for 24 hours at 4^{o}C before being used.

Sixty-four wells were cut into the agar using a cooled flamed 8 mm cork borer using a quasi-Latin square as a template. Use of the numbered template, prepared on a black card 243 x 243 mm, thereby allowed the samples to be placed randomly on the plate.

All samples were tested in quadruplicate by adding 100 μl to each of four wells on the plate. Solutions of phenol (1, 2, 3, 4, 5, 6, 7 and 8% w/v) were added to the plate as standards. A blank of distilled water (used to dilute the honey) was also included on the plate to ensure there were no contaminants.

The plates were incubated for 18 hours at 37^{o}C before placing back onto the template. Using vernier calipers, the diameter of the clear zones produced were measured and recorded in mm. The mean diameter of the clear zone around each phenol standard was calculated and squared. A standard graph was then plotted of % phenol against the square of the mean diameter of the clear zone. A best fit straight line was plotted and the equation of this line used to calculate the activity of each honey sample from the square of each measurement of the diameter of the clear zone. Antibacterial activity is therefore expressed as the equivalent phenol concentration (w/v), after allowing for the dilution of honey for the assay.

3.1.3 Results

All three honeys exhibited significant antibacterial activity (range = 18.6–23.1% phenol equivalent) when tested at 25% but not at 10% using the well diffusion assay (See Table 3.1).

Both skim milk and whole milk had no detectable antibacterial activity when assayed at full strength (see Table 3.1). This was as expected, as milk powders prepared by NZMP consist of spray-dried, pasteurised skim/whole milk. The heating of the proteins in this process destroys the antibacterial factors in the milk.

The dilution of honey in milk, to a final honey concentration of 25%, also had no effect on the levels of antibacterial activity (Table 3.1). Small variations occurred in the final percentage phenol equivalents, but these were all within the standard deviation range. No antibacterial activity was observed when the honeys were diluted to 10% in the milk samples or in water, this agreeing with earlier work that has demonstrated that the minimum inhibitory concentration of *S. aureus* is greater than 10% (Cooper *et al*., 1999).

Table 3.1: *The antibacterial activities of three different honeys (RE7, HD19 and PS3) alone and when diluted in 100% reconstituted whole or skim powdered milk. All values are mean ± SD. nd = no detected activity.*

Honey Sample	Honey concentration in assay (%)	Diluent	% Phenol Equivalent
None	0	Water	nd
None	0	Whole milk	nd
None	0	Skim milk	nd
RE7	25	Water	23.1 ± 0.7
	25	Whole milk	23.4 ± 0.5
	25	Skim milk	22.8 ± 0.4
RE7	10	Water	nd
	10	Whole milk	nd
	10	Skim milk	nd
HD19	25	Water	18.6 ± 0.6
	25	Whole milk	18.9 ± 0.2
	25	Skim milk	18.3 ± 0.7
HD19	10	Water	nd
	10	Whole milk	nd
	10	Skim milk	nd
PS3	25	Water	21.4 ± 0.4
	25	Whole milk	21.4 ± 0.8
	25	Skim milk	23.9 ± 0.5
PS3	10	Water	nd
	10	Whole milk	nd
	10	Skim milk	nd

3.1.4 Discussion

The data from this small experiment suggest that the combination of milk and honey (even at ratios higher than would likely be used in a food/drink

product) has no effect on the antibacterial activity of the honey. However, whether similar findings would be obtained *in vivo* after consuming a honey/milk drink is questionable. As mentioned earlier, the main antibacterial component of honey (hydrogen peroxide) is produced by the enzyme glucose oxidase, and enzyme activity can be affected by many factors (see Section 1.3.2.1). In particular, glucose oxidase is only active when the honey is diluted (White *et al.*, 1963) and that peak levels of accumulated hydrogen peroxide occur 6–24 hours after the honey has been diluted, with no detectable hydrogen peroxide present after 24–48 hours (Bang *et al.*, 2003). It is likely, therefore, that any drink product containing honey would likely have no antibacterial activity, even after a relatively short shelf-life. The antibacterial activity of honey could be utilised, however, in products which are not diluted to a large degree. Or, rather than targeting the antibacterial activity at the level of consumer consumption, it could be used as a part of an effective system to kill bacteria at the time of packaging.

Importantly, even if the antibacterial activity of the honey was not reduced by the time it was consumed, other factors would likely reduce the effectiveness of the honey even further. The presence of milk peptides and components of other food products consumed at the same time may very well interact with the honey, the glucose oxidase or the hydrogen peroxide to reduce its antibacterial capacity. As well, despite the fact that hydrogen peroxide can freely cross cell membranes enzymes present in the gut mucosa (including catalase and glutathione peroxidase) may well degrade any hydrogen peroxide before it is able to be absorbed.

Thus, whereas these results have given some limited data to support the antibacterial effectiveness of honey in a milk-based product, additional work could be undertaken to assess the actual antibacterial capacity of honey when incorporated into yoghurts and milk drinks. In particular, it would be of interest to ascertain what levels of hydrogen peroxide can be attained in a honey/milk product, what loss of antibacterial activity occurs with increasing product shelf-life and whether these products can have *any* antibacterial effect *in vivo*. Furthermore, the results demonstrate that there is a dilution effect of honey, as 25% solutions showed antibacterial activity whereas 10% solutions did not. It could also be of interest, therefore, to determine whether this dilution effect is dose-dependant and whether this might have any impact on the levels of hydrogen peroxide produced after consumption.

3.2 ANTIOXIDANT ACTIVITY

3.2.1 Introduction

The antioxidant capacity of honey has been demonstrated in both *in vitro* and *in vivo* studies (see Section 1.4), and it has been claimed to be correlated with the colour of the honey sample; the darker the colour of the honey, the greater the level of antioxidant activity.

Importantly, the antioxidant content of honey is one of the key properties that make it a good potential choice as a replacement for other sugars in food products. One of the big problems affecting Western populations is the increasing prevalence of Diabetes and hyperglycemia (see Chapter 9). In particular, recent research has shown that one of the ways that prolonged high blood glucose levels can have detrimental effects is through the induction of oxidative stress (an increase in oxidant production that adversely affects cellular physiology and function; see Section 9.1). This, in turn, has been shown to contribute to the pathological manifestations of several diseases and disorders, particularly those in older age. There is now a lot of evidence to suggest that cognitive decline in older individuals is correlated with the degree of oxidative damage that occurs both short- and long-term, and that antioxidant intake (dietary or supplementary) may attenuate this damage (see Chapter 10). For this reason, it was deemed relevant to assess whether milk would alter the antioxidant activities of different honeys when the two ingredients were combined.

3.2.2 Methods

The assay for antioxidant activity was that described by Molan (2003).

3.2.2.1 Sample Preparation

The same three honeys used in the antibacterial assay were used in this study. Of the three, two were dark coloured honeys (RE7 and HD19) and one was light in colour (PS3). The honey samples were diluted in deionised water to a concentration of 5% (v/v). They were then mixed well using orange sticks until all the honey was dissolved. Samples of pasteurised skim and whole milk (5%) were also prepared by diluting with deionised water.

A number of honey/milk solutions were then prepared to contain 5% honey, 5% milk (final concentrations) and 90% deionised water. All solutions were stored away from the light until assayed, and were kept for no longer than 30 minutes once prepared.

3.2.2.2 Reagent Preparation

Preparation of ABTS Cation Radical

A standard ABTS solution (7 mmol/l) was prepared by dissolving 38 mg of ABTS powder in 10 ml deionised water. To allow the formation of the $ABTS^{\bullet+}$ cation radical, 6.5 mg of potassium persulphate was added to the ABTS solution (final concentration = 2.45 mmol/l) and it was left to react in the dark for 12-16 hours (a blue-green stable free radical was formed).

Prior to commencing the assay, the $ABTS^{\bullet+}$ solution was diluted for assay. Fifteen microlitres of $ABTS^{\bullet+}$ was added to a well of a microtitre plate and diluted with 85 μl deionised water. A further 100 μl deionised water was then added in place of the sample. An endpoint protocol reading was run on a Biorad 550 microtitre plate reader at 655 nm and the optical density (OD) recorded. If the OD readings fell within the range 2.0–2.4 the solution was at the appropriate concentration. If outside this OD range, the amounts of $ABTS^{\bullet+}$ and water used were altered accordingly.

Preparation of Trolox Standard

A 2.0 mmol/l stock solution of Trolox was prepared by dissolving 25.0 mg of Trolox in 50 ml HPLC grade 95% ethanol. The stock solution was kept for a maximum of 2 days. When required, the stock solution was diluted 1:9 with ethanol.

3.2.2.3 Assay

Using a NUNC 96 well microtitre plate, each sample was plated out using 100 μl/well in rows C–H (8 replicates of one sample per row). Deionised water was used as a blank and placed in row A, and the Trolox standard was placed in row B. The plate was then read using a Biorad 550 microplate reader (endpoint protocol at 655 nm with 9 seconds shaking). This data was termed the “blank” data.

ABTS solution (100 μl) was then added to every well on the plate in a stepwise manner, starting with column 1 using a multichannel pipette. ABTS was added to each new column every 10 seconds. A second endpoint reading (as above) was then performed (termed the “endpoint” data) and the total time taken to fill the plate and to start the second plate reading recorded.

Immediately after the endpoint data was recorded, a kinetic protocol was started (655 nm, no shaking time, 5 readings to be taken at 25 second intervals).

The negative velocity was calculated by the Biorad software, and the velocity report saved.

3.2.2.4 Calculation of Antioxidant Activity

The antioxidant content of the samples was determined by taking the data generated by the plate reader and opening it in an Excel spreadsheet to calculate the following:

1. True zero-time endpoint:

   True endpoint = average endpoint + [average velocity x time for slow reaction]

2. Difference between endpoint and sample blank

   Colour of ABTS remaining = true endpoint – OD of blank

3. Scavenging capacity of sample

   Scavenging = (OD of ABTS at start) – (OD of ABTS at true endpoint)

4. TEAC (Trolox-equivalent antioxidant capacity) value

$$\text{TEAC} = \frac{\text{Scavenging at true endpoint}}{\text{Scavenging by Trolox standard}} \times \text{Trolox conc x honey/milk dilution}$$

3.2.3 Results

The antioxidant capacity of honey, milk and honey/milk solutions is given in Table 3.2. The TEAC values of the three honeys differed from each other, with the lighter coloured PS3 sample having the lowest TEAC values. Both the whole milk and skim milk samples had TEAC values < 1.0 mmol/l.

When samples of honey and milk were assayed together, an additive effect was observed. In all cases, the TEAC value obtained was almost exactly that of the milk plus honey combined. No interference was observed in any of the samples.

In further work (data not shown) the honey was diluted to 10% in milk (as 10% is the highest level of honey likely to be used in a commercial milk product) before diluting further 1:20 with deionised water for assay. These samples also showed no interference.

Table 3.2: *Mean antioxidant capacity (TEAC; ± SD) of honey, milk and honey/ milk solutions. All samples were diluted to 5% with deionised water for assay.*

Sample	TEAC value (mmol/l)
Water	0.03 ± 0.01
Whole milk	0.84 ± 0.06
Skim milk	0.81 ± 0.07
HD19	2.89 ± 0.12
HD19 + whole milk	3.78 ± 0.0.8
HD19 + skim milk	3.68 ± 0.11
RE7	2.66 ± 0.05
RE7 + whole milk	3.55 ± 0.14
RE7 + skim milk	3.51 ± 0.04
PS3	1.43 ± 0.14
PS3 + whole milk	2.29 ± 0.07
PS3 + skim milk	2.24 ± 0.06

3.3 DISCUSSION

As the data from this experiment show, milk and honey together showed an additive effect of antioxidant activity. Interestingly, the milk samples demonstrated levels of antioxidant activity that were 20–50% of those seen by the honey samples. These milk antioxidants (including cysteine, ascorbate, alpha-tocopherol, and beta-carotene) are often considered to be significant dietary contributors, particularly in light of the fact that the consumption of fresh fruit and vegetables has decreased in recent years (Zulueta *et al.*, 2007).

As with the antibacterial activity discussed earlier, the findings from this small study are limited as it does not take factors such as product formulation, shelf-life or post-consumption antioxidant degradation into account. However, research has demonstrated that antioxidants are stable during storage of up to 6 months (Di Mambro and Fonseca, 2007) or even when heat-treated at 60–100^{o}C (Xiong *et al.*, 2006). Thus, it is likely that the antioxidant content of a honey/milk based product would retain substantial antioxidant capacity, even after long-term storage.

More importantly, antioxidants are well known to be absorbed from the gut, with serum antioxidant levels peaking 2–4 hours after consumption (Mazza *et al.*, 2002; Tesoriere *et al.*, 2004; Walton *et al.*, 2006). Specifically, intake of

antioxidant-rich honey has also been shown to increase serum antioxidant levels in humans (Al Waili, 2003c; Gheldof *et al.,* 2003; Schramm *et al.,* 2003). Thus, honey does appear to have the potential to have antioxidant effects *in vivo*. A full discussion of the role of antioxidants and oxidative damage, as well as the potential effects that antioxidants originating from a honey/milk based diet may have are explored in Chapters 9 and 10.

Chapter Four:

The Effect of Honey versus Sucrose and Mixed Sugars on Protein Uptake and Protein Metabolism in Weanling Rats

This chapter presents the data from a protein evaluation study that was designed to ascertain whether feeding honey, mixed sugars or sucrose would have differential effects on the levels of protein uptake and protein metabolism in young rats. This trial is one of the three trials carried out at Crop and Food Research in Palmerston North. It was undertaken with limited internal funding, and therefore analyses were limited.

4.1 INTRODUCTION

Protein supplements are a multi-million dollar industry, providing nutritional support for the elderly (Milne *et al*. 2002, Bonnefoy *et al*. 2003; Ewans, 2004; Milne *et al*. 2005) and the unwell (Potter *et al*. 2001, Poustie *et al*. 2003). In addition, one of the largest markets for protein supplements is that providing for those wishing to increase fitness and body mass (Wolfe, 2000; Armsey and Grime, 2002; Phillips, 2004). Traditional recommendations for building lean

muscle mass have been to increase the level of resistance training and to consume more protein, however, relatively recent research now demonstrates that increasing protein intake alone is often not sufficient to replenish the protein lost after extensive or resistance exercise (Stevenson *et al*., 2005a).

Instead, research suggests that a combination of protein and carbohydrate together may actually be more effective at reducing muscle loss post-exercise because of the increased insulin response that results due to the carbohydrate content (Betts *et al.,* 2007). Furthermore, a number of studies have demonstrated that post-exercise carbohydrate intake (particularly those with a lower GI value) alone (*i.e.* without supplemental protein) can improve the rate of recovery (Stevenson *et al*., 2005a, 2005b), and that net muscle protein balance (protein synthesis minus protein breakdown) following resistance exercise is improved when carbohydrate is consumed after the period of exercise (Borsheim *et al*., 2004). It has been suggested that this results due to a decrease in muscle protein breakdown because of stabilised insulin levels (Borsheim *et al*., 2004).

Whilst most commercially available protein supplements do contain a source of carbohydrate; many of them (particularly those targeted towards body building) often have high levels of artificial sweeteners such as aspartame and saccharin added rather than natural sugars (Anderson and McMurray, 1998). This has occurred concomitantly with the move away from the use of simple sugars (including glucose and fructose) in body building supplements as public awareness has increased as to the caloric content of sugar and the detrimental health effects that excessive sugar consumption can have. Artificial sweeteners have no caloric value, though, thus they tend to produce only a negligible insulin response. As well, whereas they are generally considered to be safe, recent evidence is now suggesting that their use may be linked with the development of cancer (Bryan *et al*. 1970; Schoenig *et al*. 1985; Ellwein and Cohen, 1990; Rewick, 1990; Garland and Cohen, 1995), migraines/headaches (Lipton *et al*. 1989; van den Eeden *et al*. 1994; Schiffman, 1995), muscle tremors, vision problems and mental confusion (Anon, 1994). For this reason, there is a need to develop new protein supplements that are both healthy and nutritious, and capable of improving net protein muscle balance after illness or exercise.

As mentioned in Chapter 1, honey is a naturally occurring sweetener that contains a mix of both simple and complex sugars, as well as vitamins, minerals, acids and enzymes (Molan, 1996), substances which may all aid individuals taking protein supplements. The high glucose content of the honey (White, 1975a), in particular, may aid in protein anabolism by inducing an insulin

response that would reduce the catabolic state. However, there appears to be no literature available detailing whether honey can indeed affect protein absorption from the gut or protein metabolism.

This chapter therefore describes a simple nitrogen (N) balance study to investigate whether there are any differences in protein uptake and utilisation in rats fed diets containing sucrose and honey. A third diet group containing mixed sugars (at concentrations found in honey) is also included in this trial to determine what effects, if any, in the honey-treatment group are due to the different sugar composition in the honey diet or possible insulinmimetic effects of hydrogen peroxide. The main endpoints included in this study are the proportion of absorbed N that was retained (biological value), the proportion of ingested N that was absorbed (protein digestibility) and the proportion of ingested N that was retained (net protein utilisation (NPU)).

4.2 MATERIALS AND METHODS

4.2.1 Experimental Animals

Weaned, Sprague Dawley male rats (21-23 days, 45-50 g) were used in this feeding trial and were bred at Crop & Food Research. The trial was carried out at Crop and Food Research with full ethical approval (see Chapter 2).

4.2.2 Experimental Diets

A rewarewa honey *(Knightia excelsa)* with a high antioxidant content (TEAC = 2.7 mmol/l) content and a high level of hydrogen peroxide-induced antibacterial activity (equivalent to that of 23.4% w/v phenol; Allen *et al.,* 1991) was used in this study. A sugar profile of this honey was determined using an in-house Gas Liquid Chromoatography method at Agriquality, Auckland, New Zealand and the composition of the "mixed sugar" diet prepared such that it contained the same proportion of sugars as the original honey sample (refer Table 4.1).

Three experimental diets were prepared to contain 48% sucrose (diet #1), 48% sugar mix (diet #2) or 60% honey (diet #3) as described in Busserolles *et al.* (2002) (See Table 4.1). Full details on the dietary ingredients is given in Chapter 2. A fourth diet, containing egg albumin (Zeagold Products, Auckland, New Zealand) was prepared to contain 25 g crude protein per kg of diet as outlined in McDonough *et al*. 1990. This diet differs from the other three diets, but was formulated to provide the minimal level of protein required to sustain the life of a rat. The formulation of this di*et al*lows the metabolic faecal N output and

endogenous urinary N output values required for calculation of other parameters to be determined.

Table 4.1: *Composition of Experimental Diets (g/kg)*[a]

Ingredient	Sucrose (Diet # 1)	Mixed Sugars (Diet # 2)	Honey (Diet # 3)	Egg Albumin (Diet # 4)
Casein	245	245	245	0
Egg Albumin	0	0	0	25.2
Corn Oil	50	50	50	99.8
Sucrose	480	0.3	0	0
Fructose	0	265.4	0	0
Glucose	0	163.9	0	0
Maltose	0	19.8	0	0
Maltodextrin	0	30.6	0	0
Honey	0	0	600	0
Cellulose	50	50	50	50
Starch	0	0	0	725
Mineral Mix	50	50	50	50
Sugar-free Vitamin Mix*	5	5	5	0
Standard Vitamin Mix*	0	0	0	50
Water	120	120	0	0

* The vitamin mixes were prepared as reported in Chapter 2.1.3. The vitamin content is equivalent in both 5g of sugar-free and 50g of standard vitamin mix.
[a] Supplier information for all ingredients is given in Chapter 2.

4.2.3 Experimental Procedures

The experimental procedure used was based upon a standard protein/N evaluation study (McDonough *et al*. 1990), with the balance period extended to 10 days to allow for greater measurable differences between treatments.

Twenty-four weanling rats (8 per treatment) were housed individually in metabolism cages and offered the experimental diets at approximately 15 g/day for a pre-balance (adjustment) period of four days and a balance (measuring) period of 10 days. Water was available to the rats at all times. Live weights were recorded at the start of the pre-balance period and at the start and finish of the

balance period. During both pre-balance and balance periods, the total daily food intake for each rat was recorded, and during the balance period alone, total faeces and urine for each rat were collected daily and stored at -20°C until prepared for analysis. Urine was collected through mesh floors into containers with 0.5 ml 1 mol/l HCl.

Diets were prepared as reported in Section 2.1.3, although the vitamin mix for the egg albumin diet differed slightly from the other diets. This diet was prepared to contain 39.57 g of vitamins plus 960.43 g of sucrose and added to the diet at a level of 50 g/kg.

At the completion of the trial, faeces were freeze-dried, sieved to remove any spilled diet, finely ground, and equilibrated in open, tared, sealable Minigrip bags. Urine was filtered through dried, pre-weighed filter papers into tared plastic bottles to remove spilled diet. The urine containers and filter papers were washed and the washings combined with the urine filtrates. Total faeces and urine were weighed, and all samples (including diets) were analysed for total nitrogen by the Nutrition Laboratory, Massey University, Palmerston North, using method TN04-303 (Leco, Total combustion method). Total Energy of the diet samples was also determined by Massey University (using bomb calorimetry) and dry matters were measured as detailed in Chapter 2. This data is presented in Table 4.2.

Table 4.2: *Nutritional composition of the experimental diets*

Ingredient	Total N (%)*	Gross Energy (kJ/g)*	Dry Matter (%)
Sucrose (Diet #1)	3.41	16.00	80.95 ± 0.5
Mixed sugar (Diet #2)	3.38	15.73	79.30 ± 0.2
Honey (Diet #3)	3.52	16.33	80.35 ± 0.2
Egg Albumin (Diet #4)	0.34	17.54	85.85 ± 0.4

* These are mean values supplied by the Nutrition laboratory at Massey Univeristy, no standard deviations were supplied.

4.2.4 Statistical Analyses

One-way Analysis of variance (ANOVA) was carried out on all parameters to determine whether there were statistically significant differences between the three experimental diets. Where statistical significance was observed, post-hoc Turkey's analyses were carried out. All analyses were performed using Genstat and/or SPSS version 12 statistical software (GenStat, 2003).

4.3 RESULTS

A statistical analysis of the data revealed that there was an outlier in treatment #3 (the mixed sugar diet), this animal demonstrating lower weight gain and food intake. Given that there were no obvious signs of distress or sickness, the data for this animal are included in the statistical analysis. As a result, the variance values for this treatment group are considerably higher than expected.

4.3.1 Weight gain and Food Intake

Rats fed the diet containing honey had a significantly lower percentage weight gain that those fed the diet containing sucrose (see Table 4.3). Mean food intake and mean dietary N intake were also significantly lower in honey-fed rats compared with those fed the sucrose diet (see Table 4.3). No differences in the food conversion ratio (food intake / weight gain) were observed between the three treatment groups.

4.3.2 Urinary and Faecal Output

Mean faecal N outputs were significantly higher, and mean urinary N outputs significantly lower, in rats fed the honey-based diet compared with those fed the sucrose- and mixed sugar-based diets (see Table 4.3).

4.3.3 Nitrogen Balance Data

Honey-fed rats demonstrated the highest mean apparent N balance and true N balance values (significant vs. mixed sugar-fed rats only; $p \leq 0.01$, Table 4.3). In addition, rats fed honey excreted a higher proportion of ingested N in the faeces, and a lower proportion in the urine compared with those fed sucrose- and mixed sugar-diets (see Table 4.3). No differences were observed between rats fed sucrose and mixed sugars.

4.3.4 Biological Value, Protein Digestibility and Net Protein Utilisation (NPU)

Mean biological value, faecal protein digestibility and NPU values were significantly different in rats fed the honey-based diet compared with both other dietary treatments (see Table 4.3), indicating that honey decreased protein uptake and utilisation. Mixed sugar- and sucrose-fed rats also demonstrated significantly different mean faecal protein digestibility values from each other, but not compared with the honey-fed rats (Table 4.3).

4.4 DISCUSSION

As the results of this study demonstrate, the replacement of sugar with the equivalent amount of honey in the diet has a marked effect on weight gain, protein digestion, absorption and protein utilisation. Weight gain and food/N intake were all significantly reduced in rats fed a honey-containing diet compared with those fed a sucrose-containing diet, suggesting that the honey diet was either less palatable or may have had a satiety effect..

Differences in weight gain and food intake, although not statistically significant, were also lower in honey-fed rats than in those fed the mixed sugar diet. No measures were available as to whether the decreases in animal weight were due purely to decreased food intake or to reductions in body fat or muscle, but other research suggests that rats gain more weight and more brown adipose tissue when fed sucrose compared with an equivalent fructose-containing diet (Kanarek and Orthen-Gambill, 1982). As honey contains on average 40% fructose (accounting for ~50% of the total sugars) it is possible, therefore, that the reduced weight gain wa due to reductions in fat stores, rather than loss of muscle weight.

Table 4.3: *Assessment of experimental endpoints in rats fed the experimental diets.*

Ingredient	Units	Sucrose (Diet #1)	Mixed sugars (Diet #2)	Honey (Diet # 3)
Weight Gain	%/10 days	153.0 ± 8.0	146.9 ± 24.9	131.7 ± 11.9*
Food Intake	g/10 days	145.2 ± 5.4	139.4 ± 12.0	136.5 ± 5.0*
FCR[a]		2.7 ± 0.2	2.8 ± 0.3	2.8 ± 0.2
Dietary N intake (DNI)	mg/10 days	4805 ± 143	4697 ± 408	4651 ± 126*
Faecal N output (FNO)	mg/10 days	260.1 ± 57.0	177.3 ± 25.1**	379.8 ± 121.3*†
Urinary N output (UNO)	mg/10 days	2216 ± 246	2410 ± 183	1763 ± 317**†
Apparent N balance	mg/10 days	2329 ± 265	2109 ± 312	2508 ± 195ψ
True N balance	mg/10 days	2479 ± 268	2256 ± 322	2648 ± 194 ψ
FNO/DNI*100	%	5.4 ± 1.1	3.8 ± 0.4	8.1 ± 2.5**†
UNO/DNI*100	%	46.2 ± 5.3	51.5 ± 3.6	37.9 ± 6.4*†
Biological value	%	53.2 ± 5.2	48.6 ± 3.7	60.7 ± 54.2** ψ
Protein digestibility	%	96.9 ± 1.1	98.5 ± 0.4**	94.1 ± 2.5*†
Net protein utilisation	%	51.6 ± 4.9	47.9 ± 3.7	56.0 ± 4.8*†

* $p < 0.05$, ** $p < 0.01$ *vs* sucrose diet
ψ $p \leq 0.01$, † $p < 0.005$ *vs* mixed sugars diet
[a] Food conversion ratio (FCR) is measured as food intake divided by weight gain.

However, removal of the outlier from the mixed sugars treatment group (the outlier was determined using a preliminary REML analysis) increased the mean weight gain from 146.9 to 151.5 %/10 days, and the mean food intake from 139.4 to 141.3 g/10 days. Weight gain and food intake are then similar for rats fed 48% sucrose and 48% mixed sugars, and both groups differ significantly from the honey-fed rats. This latter data would suggest that the reduction in weight gain and food intake in honey-fed rats was not simply due to the different sugar composition of the diet, but may have been due, in part, to the other components of the honey. It is possible that the intake of honey may have had a physiological effect on the stomach. Goldschmidt and Burkert (1955), for example, have reported that honey acts as if it contains acetylcholine and choline esters in it which could effect the gastric muscle. Other hormonal or neurotransmitter activities are also possible as a result of consuming honey, although nothing has been reported in the literature to support this.

The results of this study also show that the type of sugars added to the diet can have an impact on the level of protein absorption and utilisation. Honey-fed rats, for example, absorbed a significantly lower proportion of ingested N, suggesting reduced digestion. Approximately 8% of the ingested N was excreted in the faeces of these rats, compared with 5% and 4% for rats fed sucrose and mixed sugars. This decreased absorption may also account for the reduced weight gain seen in honey-fed rats.

One possible explanation for the reduced amino acid absorption is the presence of tannins (astringent polyphenol compounds) in honey. Condensed tannins present in forage crops have been shown to inhibit protein digestion in sheep (Egan and Ulyatt, 1980; Barry *et al*., 1986; Messman *et al*., 1996; Barahona *et al*., 1997; Barry and McNabb, 1999), but as rats do not generally eat forage crops, corresponding data for this species are not available. The presence of tannins in honey have not been reported in the literature, although given that honey is a concentrated plant fluid it is not unreasonable to expect that they could occur. Indeed, Yilmaz *et al*. (2003) demonstrated the presence of polyphenol oxidase (an enzyme responsible for the oxidation of mono- and di-phenols) in Turkish honeys. These authors report that the presence of polyphenol oxidase appears to correlate with particular floral sources, although no information is available as to which floral factors may be involved, or whether these factors may have been present in the New Zealand Rewarewa honey used in this study. However, it has also been reported that antioxidants in honey can inhibit the activity of polyphenol oxidase in fruit and vegetable homogenates by

2–45% (Chen *et al*. 2000). This would suggest that any possible tannin production in honey may be reduced due to the presence of antioxidants, although no information is available as to whether tannins, once produced, are stable in honey. It would be of interest to investigate and quantify the presence of polyphenol oxidase and/or tannins in honeys of various floral sources.

Protein digestion may also have been reduced in honey-fed rats because of the presence of protease inhibitors. Honey has been shown to contain several enzymes (including glucose oxidase, amylase, invertase, catalase and acid phosphatase) as well as other protein constituents (White, 1975a, 1975b) and some of these appear to be regulated by enzyme inhibitors present in the honey. Glucose oxidase, for example, is virtually inactive in full strength honey, with activity increasing 2,500 – 50,000 times as honey is diluted (White *et al*., 1962). Inhibition of the other proteases in honey does not appear to have been investigated, although unpublished work in our laboratory has demonstrated that lactoperoxidase and horseradish peroxidase are both rapidly inactivated by honey at concentrations as low at 3%. Thus it is possible that enzyme inhibitors present in honey may have an impact in the gut after being consumed.

A further possibility for the reduced N absorption observed in honey-fed rats is a prebiotic effect. Several authors have demonstrated that honey has a prebiotic effect *in vitro* (Chick *et al*., 2001; Ustunol and Gandhi, 2001; Kajiwara *et al*., 2002; Sanz *et al*., 2005) and this is discussed in Detail in Section 1.6. This hypothesis of a prebiotic effect is supported by the fact that the mean faecal N output was significantly increased in honey-fed rats (380 mg) compared with rats fed sucrose (260 mg) and mixed sugars (177 mg). However, faecal N output was significantly lower in rats fed mixed sugars compared with those fed sucrose. A prebiotic effect involves the available sugars being used by the gut bacteria rather than being absorbed. Rats fed the mixed sugars should therefore have faecal output values that were similar to rats fed honey, and greater than rats fed sucrose. Clearly, the data does not show this, thus the idea of a prebiotic effect is questionable. More than likely, the differences in N absorption were due to a number of different factors.

Importantly, high protein supplements are usually taken in an attempt to improve total or lean muscle mass, and despite the fact that faecal N output was higher in honey-fed rats, the N balance (total N intake minus total N output) remained higher in these animals compared with those fed sucrose. Indeed, despite the apparent reduction in protein digestion and absorption, honey-fed rats

demonstrated significantly higher biological values and net protein utilisation values suggesting that what protein was absorbed was better utilised.

That the altered N balance occurred in rats fed honey but not in those fed mixed-sugars suggests that this too is due primarily to the non-sugar components of the honey. One explanation is the presence of hydrogen peroxide in the honey, as research *in vitro* has shown hydrogen peroxide to be a strong insulin-mimetic agent (Czech *et al*., 1974; Hayes and Lockwood, 1987; Heffetz *et al*., 1990). Levels of hydrogen peroxide production in honey vary depending on floral source *etc*, but were shown to be in the range of 0.5–3.5 mmol/l in high peroxide-producing honeys after prolonged incubation (Bang *et al*., 2003). Although not measured using the same method, the Rewarewa honey used in this study has also been shown in our laboratory to have a similar level of hydrogen peroxide production. These levels agree with the 1–3 mmol/l shown to induce tyrosine phosphorylation (an insulin-mimetic effect) *in vitro* (Hayes and Lockwood, 1987; Heffetz *et al*. 1990), although hydrogen peroxide levels in honey would arguably be considerably less *in vivo* after dilution the gut. However, there is also quite a lot of hydrogen peroxide produced metabolically in the body, although most it is usually broken down in vivo by the Fenton reaction (Winterbourn, 1995). Recent work has shown that Honeydew and Rewarewa honey is very good at inhibiting the Fenton reaction (Brangoulo and Molan, 2008), thus it is possible that the intake of honey could increase the levels of circulatory hydrogen peroxide by preventing its decomposition. As excess hydrogen peroxide is excreted in the urine, it could be of interest to measure levels in human urine before and after eating Honeydew or Rewarewa honey to ascertain whether they can alter the circulatory levels of hydrogen peroxide *in vivo*. If higher levels of hydrogen peroxide do result after eating honey, it could therefore be possible that this leads to enhanced glucose transport (via activation of insulin receptors by hydrogen peroxide) and utilisation (*i.e.* more stable blood sugar levels), and increased protein anabolism (although the mechanisms by which this may occur are unknown).

Unfortunately, due to financial constraints a number of important measures could not be performed. Certainly, it would have been valuable to have body composition data to assess whether differences in weight gain were due to differences in fat or protein. As well it would have been of interest to evaluate insulin responses after intake of the sucrose, mixed sugars and honey diets. These measures should definitely be considered if this work is to be continued and expanded upon.

In addition, this trial was designed with sugar or honey as the sole carbohydrate source in the diet, and such a dietary regimen is unlikely to occur in real life. Instead, protein supplements are generally incorporated into the diet, along with other proteins, fats, carbohydrates *etc*. Studies of the effects of honey at substantially lower concentrations on protein metabolism are therefore warranted, particularly with reference to interactions with other dietary constituents.

Lastly, as mentioned earlier in the chapter, the diet composition of the honey, sucrose and mixed sugars diets were based upon those of Busserolles *et al.* (2002) and the protein concentration used in the experimental diets was 24.5%. A closer review of the literature suggests, though, that standard analyses of biological value (BV), net protein utilisation (NPU) and protein digestibility are performed using a protein concentration of 10% (James *et al.,* 2003). This is a fairly major flaw in the design of this study, and, as such, the results generated must be considered with this in mind. However, given that the results of this study were only compared within the study (*i.e.* between treatments) and not with data obtained from other BV/NPU studies, the comparative findings can still be considered to be valid results into themselves, and indicative of differences between sucrose, mixed sugars and honey. Of course, further studies will need to be undertaken, using the correct protein levels, before any true conclusions as to the impact of honey and sucrose on protein utilisation can be drawn.

Chapter Five:

The Weight Loss Potential of Honey versus Sucrose and Mixed Sugars in Adult Rats

This chapter presents the data from an 8-week feeding study that was undertaken to assess whether honey, sucrose or mixed sugars as in honey would have differential effects on weight gain in adult rats. The trial is one of the three trials carried out at Crop and Food Research in Palmerston North. It was carried out in 2004, approximately 16 months before the conception of this thesis, and thus was done as a stand-alone study. This trial was funded by internal funding from Crop and Food. Therefore, the types of analyses employed in this study were limited due to financial constraints.

A second study assessing the effects of honey on weight was carried out at Waikato University during the time of this thesis (see Chapter 6). Given that these two trials are related, a brief discussion of the current trial will be given in this chapter, with a more detailed discussion of the effects of honey on weight gain and/or loss given at the end of Chapter 6.

5.1 INTRODUCTION

Obesity is a major concern for westernised populations, and in the USA more than 133 million people (approximately 60%) are now considered to be overweight or obese (National Center for Health Statistics,. 2006). Surveys consistently show that adults are concerned by weight, and the health-related implications it incurs, and that most are making a concerted effort to either maintain or lose weight (Serdula *et al.* 1999; Scott *et al.*, 2006). Despite this, however, the end result is that the proportion of individuals who are overweight or obese has increased steadily over the last 30 years. As such, obesity is now a major threat to health and quality of life. In Western countries, excess body fat is responsible for 30–40% of coronary heart disease, colon, breast and endometrium cancers as well as most cases of Diabetes (Saris, 2003).

The increase in weight observed in western society is a complex issue, although reasons includes factors such as increased workloads/stress and reduced levels of exercise. The largest contributor, however, is thought to be the change in diet that has occurred in the last quarter century. During this time convenience and “junk” foods have become readily available and they now often constitute a large part of the weekly food intake. These foods are generally highly processed, low in nutrition, and often contain excessively high levels of sugar and fat.

In the 1970s, many nutritionists believed that sucrose was the most important predisposing factor for weight gain (Yudkin, 1986), but this idea was quickly replaced with the suggestion that dietary fat was the most important nutritional component responsible for weight gain and excess energy intake (Flatt, 1987; Astrup, 1999). The idea that consuming excess fat leads to increased body fat levels was quickly embraced by health practitioners as well as the general public, and health guidelines tended to focus on two particular lifestyle factors to reduce and manage obesity: increased levels of physical activity and reductions in the intake of fats (Saris, 2003).

As a consequence, the market for low-fat foods has rapidly expanded since the early 1990s (Leveille, 1997) and nutritionists have recommended a low fat, high carbohydrate diet for healthy living. Unfortunately though, despite the absolute reduction in fat in the diet over the last 10 years (Kennedy *et al.*, 1999), Western populations are still getting fatter. The direct relationship between dietary fat and obesity has again been questioned (Saris, 2003), particularly as low-fat foods are often based on sugar to maintain taste and palatability and thus

often have energy density values that are comparable to their high-fat counterparts (Willet, 1998).

Recently, the spotlight has again moved back to the importance of dietary sugars with emphasis on their effects on blood sugar responses. Research has shown that only small amounts of carbohydrate can be stored as glycogen in the human body, and that any excess must be oxidised or converted to fat by *de novo* hepatic lipogenesis (Acheson *et al.*, 1988). Intake of refined carbohydrates therefore results in postprandial carbohydrate oxidation at the expense of fat oxidation, altering fuel partitioning such that body fat gains are favoured. This data is further supported by a substantial number of epidemiological studies that have found a clear relationship between sucrose intake and body weight/body mass index, as well as sucrose intake and total fat intake (reviewed by Astrup and Raben, 1995 and Hill and Prentice, 1995). Similar findings have also been observed in animal studies, with rodents fed a high sucrose diet exhibiting higher weight and fat gains compared with those fed standard chow (Kanarek and Marks-Kaufman, 1979; Morris *et al.*, 2003; Kawasaki *et al*., 2005) or starch based diets (Hallfrisch *et al.,* 1981; Reiser and Hallfriesch, 1977). It has been suggested that the heightened levels of obesity seen with sucrose are due primarily to the elevated blood glucose and insulin levels that result after consumption (Astrup and Raben, 1995).

Glycemic index is now also being recognised as a factor that can influence satiety and hunger. In particular, foods that have a high glycemic index (*i.e.* those containing carbohydrates that are broken down quickly) tend to result in a large surge in insulin production that leads to a drop in blood glucose soon after eating. This generally results in hunger soon after the meal has been consumed, followed often by more eating. In contrast, foods with a low glycemic index tend to maintain a comfortable blood glucose lever for a longer period of time, hunger after a meal

Given the overeating and obesity that is prevalent in Western societies, foods and beverages are now often produced with a “diet” version using artificial sweeteners such as aspartame and saccharin (Anderson and McMurray 1998). However, research has demonstrated that these too may be associated with negative health impacts including cancers (Bryan *et al.*, 1970; Schoenig *et al.*, 1985; Ellwein and Cohen 1990; Rewick 1990; Garland and Cohen 1995), migraines/headaches (Lipton *et al.*, 1989; van den Eeden *et al.*, 1994; Schiffman 1995), muscle tremors, vision problems and mental confusion (Anon 1994).

Today, many consumers now demand foods that are healthier and, as a result, certain aspects of nutrition research are now centred on finding food ingredients that can offer nutritional advantages. Honey is recognised as having many beneficial health effects (refer to Chapter 1) and recent research suggests that it may offer advantages as an alternative to the sugars that are more commonly used in food products. In particular, honey offers the sweetness of other sugars, but with additional benefits. Some honeys have been shown to have a lower glycemic index value than sucrose (Shambaugh *et al.,* 1990; Schramm *et al.*, 2003) and it has been reported that honey, sucrose and fructose do have differential effects on blood glucose levels (Akhtar and Khan, 1989; Al-Waili, 2003c; 2004b). Furthermore, it has also been reported the rate of glucose uptake is actually slowed following consumption of a mixture of fructose and glucose where the fructose content is higher (MacDonald, 1968). Honeys usually contain more fructose than glucose (a 1.2:1.0 ratio; White *et al.*, 1975a), although the actual ratio of fructose to glucose can vary dramatically in a particular honey. The honey used in this study had a higher than average fructose content (40.9%, equivalent to 50% of the total sugars) with a ratio of fructose : honey of 1.36: 1.0, thus allowing any possible effects of reduced glucose absorption to be observed.

In addition, it has been demonstrated that rats fed a high fat/high sugar diet that also contains green tea exhibit significantly reduced weight gain compared with those animals fed the diet without the green tea (Choo *et al*., 2003).The research indicates that the antioxidants that occur in the green tea are responsible for this effect as they can activate β-adrenoceptors in brown adipose tissue, thereby leading to increased thermogenesis of the adipose tissue (Choo *et al.*, 2003). Honey also can contain high levels of antioxidants (Frankel *et al.,* 1998; Gheldof and Engeseth, 2002), thus, it is possible, that the antioxidant content of honey may also contributed to reduced weight gain compared with animals fed the antioxidant-free sucrose-based diet.

This trial was therefore designed to ascertain what effect honey might have on the weight gain of adult rats, as compared with those fed a sucrose-based diet or a diet containing sugars in the same proportion as in honey.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Animals

Thirty 14-week old Sprague Dawley male rats (380–550 g) were used in the feeding trial. These were bred by Crop & Food Research in Palmerston North. The trial was carried out in the Food Evaluation Unit at Crop and Food Research

as described in Section 2.1.1 with full ethical approval (see Section 2.1.4). The rats had previously been used for a Protein Efficiency Ratio study; however, they had been fed standard rat pellets for 2 weeks before starting the current trial.

5.2.2 Experimental Diets

The composition of the diets in this study were based upon the standard diet formulas used to assess weight gain in rodents during commercial feeding studies at Crop and Food Research (Butts *et al.*, 2003). A honeydew honey (*from Nothofagus solandri*) with a known high rate of hydrogen peroxide production (antibacterial activity equivalent to 18.6% phenol; Allen *et al.*, 1991) and a high antioxidant content (TEAC = 2.9 mmol/l) was used for this study. A sugar profile analysis was performed using in-house GLC methods by Agriquality, Auckland, New Zealand. The data from this profile was then used to calculate the concentrations of sugars used in the mixed sugars diet (diet # 1055) such that the concentrations of the various sugars were the same as in the honey diet.

Three experimental diets were prepared to contain 8% sucrose (# 1054), 8% mixed sugars (diet #1055) or 10% honey (diet #1056) (the honey contained 20% water). A mineral mix and a sugar-free vitamin mix were prepared in-house at Crop and Food Research as described in Section 2.1.3. The diet compositions are given in Table 5.1. Sub-samples (~10 g) were taken from each diet at the start of each week and dry matter contents calculated by drying for 16 hours overnight at 105^{o}C.

5.2.3 Experimental Procedures

At 12 weeks of age the 30 rats were placed into raised stainless steel cages with 6 mm^2 mesh floors. Numbered blotter papers were placed under each cage to catch the spilled diet, faeces *etc*. Because the animals had previously been used for a protein evaluation study, a wash-out period was required before starting the animals on the experimental diets. All rats were fed the sucrose diet for 14 days to allow the animals to adjust to the new diets. Animals were weighed every 7 days but food intake was not recorded.

At 14 weeks of age, all animals were weighed and grouped into three dietary regimens, such that each treatment group had the same approximate mean body weight and coefficient of variation (COV). The animals in each treatment group were then allocated one of the three diets for 8 weeks.

Glass jars containing the appropriate diet were weighed at the start of each week and placed into the respective cages. New food was weighed and

added to each food pot as required (no more than once daily) so that food was available *ad libitum.*

Every seven days, the rats were weighed and the existing food pots removed and weighed. The blotter papers were replaced weekly and allowed to dry at 25^{o}C for 3–4 days. Once dry, all material on each blotter was placed into a standard kitchen sieve. Faeces and hair were retained, whilst the spilled diet passed through and was collected and weighed. Fresh water was provided at all times, with water bottles cleaned twice weekly.

Table 5.1: *Composition of Experimental Diets (g/kg)*[a]

Ingredient	Sucrose (#1054)	Mixed Sugars (#1055)	Honey (#1056)
Lactalbumin	238	238	238
Corn Oil	100	100	100
Sucrose	80	0.5	0
Fructose	0	40.9	0
Glucose	0	30.1	0
Maltose	0	3.4	0
Maltodextrin	0	5.1	0
Honey	0	0	100
Cellulose	50	50	50
Mineral Mix	50	50	50
Vitamin Mix	5	5	5
Starch	457	457	457
Water	20	20	0

[a] Supplier information for all ingredients is given in Chapter 2

5.2.4 Statistical Analyses

One-way ANOVA analyses were carried out on all endpoints. A (9 x 3) repeated measures ANCOVA was then performed on the weight gain data (using the total food intake as a covariable) to determine if there were any diet x time interactions. Post-hoc analyses included Turkeys analyses. All analyses were performed using Genstat statistical software (GenStat, 2003) and/or SPSS version 12.

5.3 RESULTS

All the rats appeared healthy throughout the 8 week study and no trial-related difficulties were reported. The mean actual weight gain is presented in Figure 5.1. Standard ANOVA analyses suggested that there was no difference in weight gain between the treatment groups at the completion of the trial, although actual mean weight gain was numerically higher in rats fed the sucrose-based diet (37.7%) compared with rats fed either the mixed sugars (31.1%) or honey-based diets (32.3%). However, the repeated measures ANCOVA (using the total food intake for each animal as a covariable) revealed that whilst there was no interaction of diet x time over the 8-week period, there was a significant effect of diet. Mean weights were significantly lower in honey-fed rats compared with both those fed sucrose ($p < 0.05$) and those fed the mixed sugars diet ($p < 0.05$).

Weekly percentage weight gain was similar for all three treatment groups and is given in Figure 5.2.

No statistically significant difference was observed for the overall food intake between treatment groups, although rats fed the mixed sugars diet demonstrated the lowest numerical mean food intake per 8 weeks (1263 g *vs* 1318 g and 1323 g for animals fed the sucrose and honey diets, respectively; see Figure 5.3).

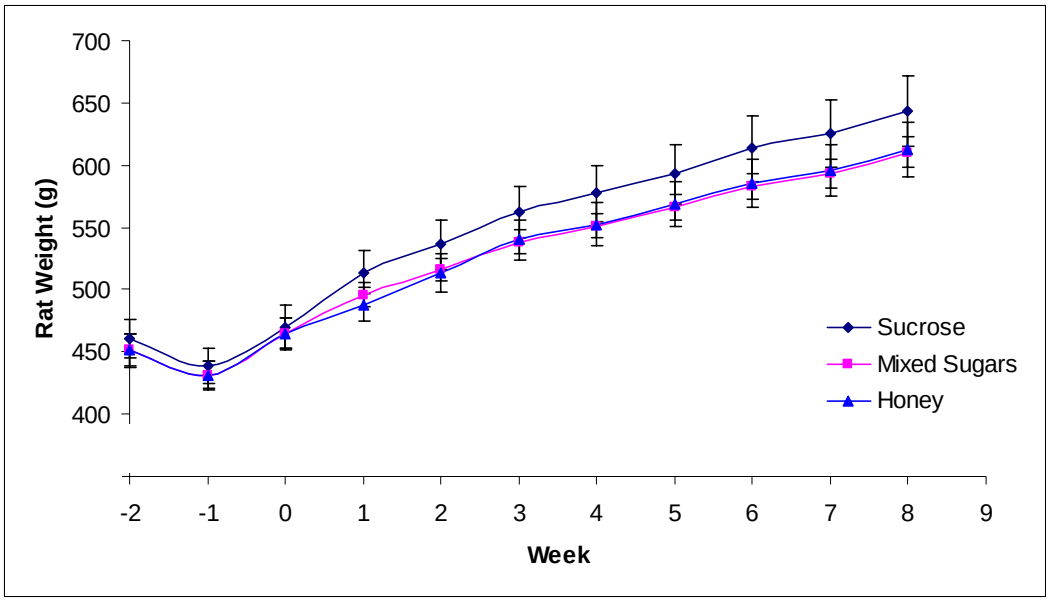

Figure 5.1: *Actual mean weight gain (± SEM) for rats fed diets containing either 8% sucrose, 8% mixed sugars, or 10% honey (honey is 20% water). Because of being used in a previous study the animals were all fed the sucrose diet for 2 weeks before starting their experimental diets. Animal weights were recorded during this time, and are presented as weeks -2 and -1 on the graph.*

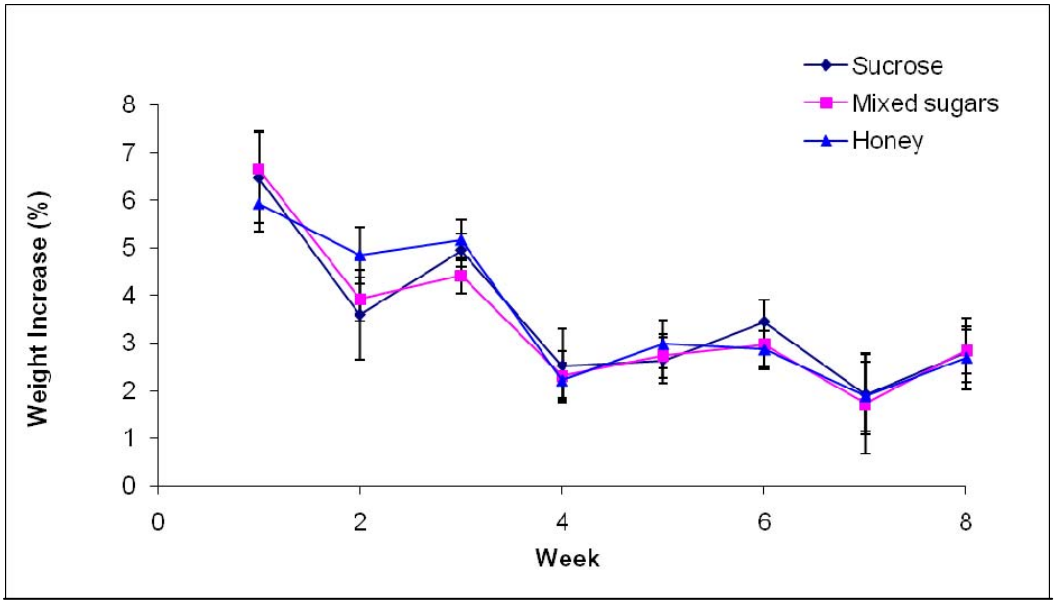

Figure 5.2: *Mean percentage weight gain (± SEM) for rats fed diets containing 8% sucrose, 8% mixed sugars, or 10% honey (honey was 20% water). (The weight gain each week is taken as the percentage of the previous weeks weight).*

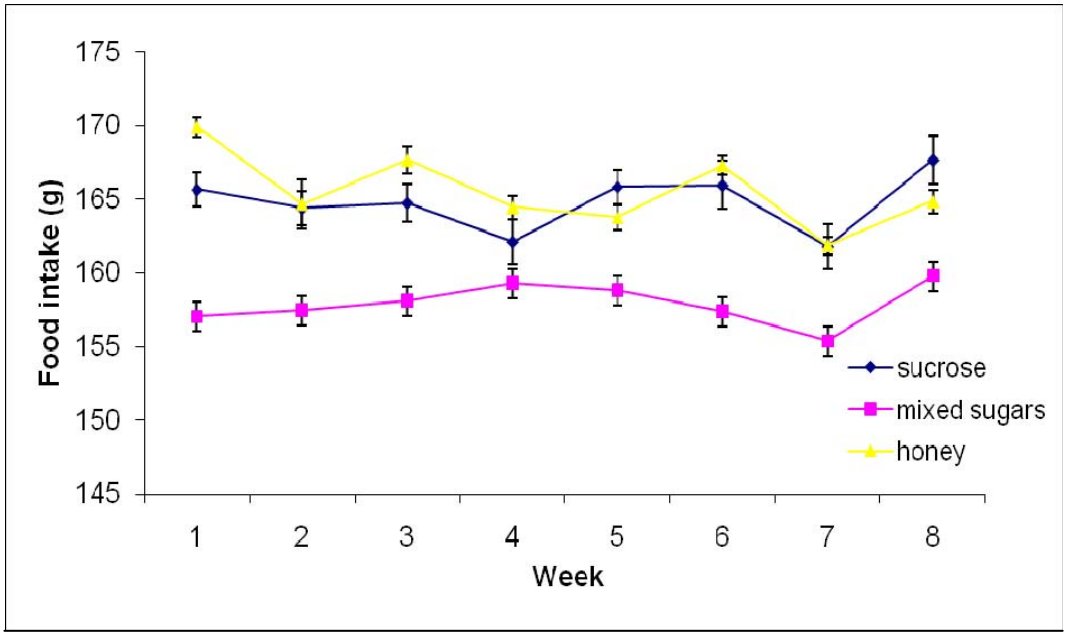

Figure 5.3: *Mean weekly food intake for rats fed diets containing 8% sucrose, 8% mixed sugars, or 10% honey (honey is 20% water).*

5.4 DISCUSSION

As the results of this small study show, there were no significant differences in levels of mean weight gain or food intake in adult rats fed diets containing either sucrose, honey or mixed sugars as in honey when using standard ANOVA analysis, although the difference between treatments reached significance using ANCOVA with food intake as a covariable.. As expected from looking at Figure 5.1, the sucrose-fed rats did gain significantly more weight than those fed honey after 8 weeks. Interestingly, though, it was also demonstrated that when the data was corrected for the numerically lower food intake in animals fed the mixed sugars diet, there was also a significant difference in weight gain between the rats fed mixed sugars and those fed honey after 8 weeks. It was initially suggested that differences in weight gain, despite similar food intake values, were due to differences in caloric intake. However, an assessment of the calorie content of each diet (measured by the Nutrition Laboratory at Massey University) revealed that the sucrose, mixed sugars and honey diets were similar (18.77, 18.60 and 18.84 kJ, respectively) and that the total caloric intake per 8 weeks was not significantly different between treatments (24745, 23496 and 24916 kJ respectively).

It is possible that significant differences in weight gain were not observed between treatments with standard ANOVA analyses because of the small number of animals used in each treatment group (n=10). Each treatment group was weight-banded such that they had the same approximate mean starting weight; however, because of a necessity to use all available animals it was not possible to reduce the intra-group weight variability. Starting weight COV values were 11.7%, 8.8% and 9.2%, respectively, for the animals fed the sucrose-, mixed sugars- and honey-based diets. In a number of other confidential commercial weight loss trials carried out at Crop and Food Research, Palmerston North, in which the same trial design was used (Bang *et al.* 2005a, 2005b) it was shown that significant differences in weight gain were only observed between treatment groups when the starting weight COV was below 5%. It is likely, therefore, that a significant difference in overall weight gain may have been observed between sucrose-fed and mixed sugars- and honey-fed rats without using total food intake as a co-variable if the intra-groups COV values had been lower at the start of the trial. However, there are costs associated with raising an excess number of animals to the required age, and this must be taken into account when designing any further weight loss trials. In addition, the variable weights of the rats used may be more representative of a population as a whole

(people, for example, can vary greatly in their actual weights for a given age). Furthermore, the animals used in this study had previously been used in another study, and it is difficult to know whether the starting weight variability seen in this study was natural or a result of the diets fed in the previous study.

As mentioned earlier, the animals in this trial were grouped to give the lowest intra-group COV and it may have been better to have fully randomised the rats to the three treatment groups. The animals were all randomised to their treatments in the previous study though the details are not available as to what the treatments were or how many experimental groups there were. It is possible, though, that by not fully randomising the treatment groups in the current study that they may have been inadvertently biased. Indeed, this may explain, in part, why rats fed the mixed-sugars diet appeared to consume less food throughout the 8-week period, leading to a significant effect seen with ANCOVA between these rats and those fed honey. Further work with rats that have not been used for previous studies will need to be done to better understand what difference in weight gain and/or food intake may occur with animals fed sucrose, mixed sugars and honey, and to further elucidate what role honey may have on preventing weight gain in adults animals.

In conclusion there does appear to be some evidence to suggest that diets based upon honey could lead to reduced weight gain in sedentary animals. Certainly, further studies need to be undertaken to assess whether this effect might be more obvious with larger sample numbers, but preliminary data from this study is unclear as to whether the lower weight gain seen in honey-fed rats is due to the sugar component of the diet or due to other minor components as the weight gain observed in animals fed honey was lower than in those fed mixed sugars when the data was corrected for food intake (suggesting that non-sugar compounds such as antioxidants could be involved), but food intake between groups was not statistically different. Limited research has also demonstrated that a single dose of honey (100 g in 250 ml water) can have a laxative effect *in vivo* (Ladas *et al.*, 1995, Ladas and Raptis 1999) because of its high fructose content (35–45%; White *et al.*, 1975a), thus it is possible that the lowered weight gain seen in honey-fed rats could be due to reduced absorption. Indeed, clinical studies have reported that healthy individuals have been shown to exhibit incomplete fructose absorption and concomitant loose stools after consuming pure fructose or fructose mixed with other sugars (Ravich *et al.*, 1983; Truswell *et al.*, 1988; Rumessen, 1992).

However, as fructose would have constituted only 4.1% of the mixed sugars and honey diets it is unknown whether such concentrations would have produced a laxative effect in these rats. In the clinical studies incomplete absorption and loose stools were reported after consuming a single dose of 50 g (Ravich *et al.*, 1983; Truswell *et al.*, 1988; Rumessen, 1992). No information was given as to patient weight, however if we assume an average adult weight of 70 kg then this laxative effect occurred at level of 0.7 g fructose / kg body weight. Animal weights in this study ranged from 450–650 g and food intake was 20–25 g (of which fructose was 0.8–1 g). The level of fructose consumption was therefore approximately 2 g/kg body weight which is higher than that reported in clinical studies. This data suggests that the concentrations of fructose in the rat diets may have been sufficient to induce reduced absorption and/or a laxative effect. Faecal output was not recorded in this study, but it was shown to be significantly higher in rats fed 60% honey (but not mixed sugars) compared with an equivalent amount of sucrose (see Chapter 4).

Fructose is absorbed via the sodium-independent transport system (Levin, 1994) and evidence suggests that it is facilitated by the absorption of glucose (Truswell *et al.*, 1988). The human small intestinal mucosa has been shown to completely absorb solutions containing equimolar concentrations of glucose and fructose (Levin, 1994; Ladas *et al.*, 1995), but different degrees of absorption occur when subjects consume pure solutions of fructose (Rumessen and Gudmand-Hoyer, 1986). Glucose concentrations can also vary considerably in different honeys (White *et al.*, 1975a), so the degree of fructose absorption may depend, in part, on the concentrations of fructose and glucose as well as their proportions relative to each other. Recent research has demonstrated that even small amounts of glucose (e.g. 10% of a 75% glucose: fructose solution) may prevent fructose malabsorption (Born, 2007). Thus it is likely that fructose malabsorption is not responsible for the decreased in weight gain seen in honey-fed rats. Instead, it is likely that other factors may be involved in the decreased weight gain observed in rats fed honey and mixed sugars, and it would be advantageous to undertake oral glucose tolerance testing in future studies. The effects of honey as a possible weight- reducing agent are discussed further in Chapter 6.

Chapter Six:

The Effect of Honey versus Sucrose, Mixed Sugars and a Sugar-free Diet on Weight Gain in Young Rats

This chapter presents the data from the 6-week feeding study that was undertaken to assess whether honey, sucrose, mixed sugars as in honey or a sugar-free diet would have differential effects on weight levels in young rats. This trial was designed to follow on from the earlier work presented in Chapter 5, but with a slightly different trial design and additional analyses, to hopefully ascertain whether statistical differences could be determined between the different dietary treatments. This trial was carried at Waikato University and was fully funded by Fonterra Brands Ltd.

The main findings from this study have been published as follows:

Chepulis, L. M. (2007) *Journal of Food Science*, 72(3): S224-S229 (see Appendix 3)

6.1 INTRODUCTION

As the results from the study in Chapter 5 suggest, honey may have an effect on weight gain in adult rats. However, weight gains in adults (animals or

people) are considerably less than during growth, thus the decision was made to investigate the effects of honey in weaning rats rather than in adult animals in this study. Further support for this decision came from the findings of Chapter 4 that demonstrated a positive effect of honey on protein metabolism in young animals.

As the study in Chapter 5 also demonstrated, it was unclear whether the differences in weight gain seen between the different groups was due to the different sugars present in the diets or due to non-sugar components such as antioxidants. For this reason the mixed sugars diet was again included as one of the dietary treatments. Honey contains a number of other compounds, and any of these could also effect absorption of nutrients, gut transit, glycaemic response *etc*. As well as the research suggesting that antioxidants may reduce weight gain in a high fat/high sugar diet (Choo *et al.*, 2003) limited research has demonstrated that hydrogen peroxide can stimulate insulin receptors (Heffertz *et al.* 1990) thereby modulating effects on blood sugar and cellular glucose uptake. Certain honeys have been shown to produce relatively large quantities of H_2O_2 once it is diluted (via oxidation of glucose by the enzyme glucose oxidase; see Section 1.3.2) although the amounts produced depend on a large number of factors including floral source, degree of handling and processing and exposure to heat and light (see Section 1.3.2.1). However, the presence of glucose oxidase, and the resultant production of H_2O_2, is endemic to most floral sources (Brady *et al.*, 2004). Thus a high hydrogen peroxide-producing honey was again used to determine what effect, if any, H_2O_2 had in the gut after consumption.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Animals

Forty rats, aged approximately 6 weeks at the start of the trial, were obtained from AgResearch, Hamilton. The trial was carried out in the small animal house (BL5) at Waikato University with full ethical approval (See Chapter 2 for full details).

6.2.2 Experimental Diets

A similar honeydew honey that was used in the study in Chapter 5 was used in the present study (hydrogen peroxide: 18.2% phenol equivalent; antioxidant content: TEAC = 2.7 mmol/l). The sugar profile analysis obtained in Chapter 5 was used to determine the concentrations of sugars added to the mixed sugar diet (diet # 3) such that the concentrations of the various sugars were the same as in the honey diet.

In this study, the diets were prepared such that they approximated the composition of a typical New Zealand diet. Based upon data from the 1997 National Nutrition Survey (New Zealand Ministry of Health, 1999), the diets were formulated so that of the total energy content, 15–16% came from protein, 35% came from fat and 45–47% came from carbohydrate.

Four experimental diets were prepared to contain no sugar (diet # 1), 8% sucrose (# 2), 8% mixed sugars (diet # 3) or 10% honey (diet # 4). All diets were prepared to contain a minimum of 4% water. Additional water was also added to each kilogram of the non-honey diets to account for the fact that the honey contained 20% water. Salt and vitamin mixes were prepared as given in Chapter 2. The composition of the diets is given in Table 6.1.

Sub-samples of the four diets were sent to the Nutrition Laboratory at Massey University for measurement of energy content. The percentage dry matter of these diets was also determined during weeks 1 and 6 by drying pre-weighed samples at 105^{o}C for 16 hours. Percentage dry weights were used to calculate actual food intake and to ensure that there were no differences in moisture content during storage.

Diets were stored at -18^{o}C until required, and allowed to warm to room temperature for 3 hours before feeding to the animals.

Table 6.1: *Composition of Experimental Diets (g/kg)*[a]

Ingredient	Sugar-free (Diet # 1)	Sucrose (Diet # 2)	Mixed Sugars (Diet # 3)	Honey (Diet # 4)
Lactalbumin	160	160	160	160
Corn Oil	150	150	150	150
Sucrose	0	80	0	0
Mixed sugars	0	0	80	0
Honey	0	0	0	100
Cellulose	50	50	50	50
Mineral Mix	50	50	50	50
Vitamin Mix	5	5	5	5
Starch	525	445	445	445
Water	60	60	60	40

[a] Supplier information of all ingredients is given in Chapter 2

6.2.3 Experimental Procedures

Animals were weighed upon receipt, and those within the starting weight range (140–150 g) were randomly allocated to 1 of the 4 dietary treatments. The remaining animals were housed in standard rat cages, with access to rodent pellets and water. These animals were weighed each morning and added into the trial once at the appropriate weight.

During the experiment, the rats were housed in standard rat cages (2 animals per cage) that had a raised mesh floor. Blotting paper was laid on the bottom of the cage (below the mesh) to absorb the urine, and collect the spilled diet and faeces. A 25 cm long piece of 90 mm PVC tubing was affixed into each cage using a metal hose clamp. This allowed the animals to get off the mesh floor to a place where they could "hide". Food, blotters and water were prepared and replaced as described in Section 5.2.3.

On day 42 after entering the trial, the rats were anaethetised using CO_2 gas. Dr Lance McLeay of the Department of Biological Sciences, Waikato University removed 3–5 ml of blood from the heart of each animal via cardiac puncture using 19 gauge needles. Approximately 1.5 ml of the removed blood was added to an EDTA-coated blood collection tube (for measurement of glycated haemoglobin (HbA1c)) and the remainder added to a standard vacutainer. These latter blood samples were allowed to clot, before centrifuging at 3000 *x* g for 10 minutes and removing the serum (for fasting lipid profiles). Both the EDTA-collected blood and the serum were then taken to the laboratory at Waikato Hospital for their respective analyses (refer Chapter 2 for details). All animals were then euthanised with CO_2 gas after the blood removal, and the bodies numbered and stored in a freezer.

After thawing, each rat was minced using a Sunmile SM-G50 mincer (Vantage Systems, Ltd.). The samples were double bagged and frozen before sending to the Nutrition Laboratory at Massey University for measurement of total body fat by Soxhlet fat extraction analyses. The rat samples were also analysed for % Protein (using a nitrogen – protein conversion factor of 6.25) by the LECO total combustion method (see Chapter 2 for details).

6.2.4 Statistical Analyses

One-way ANOVA (followed by turkey's post hoc analyses) were carried out on overall percentage weight gain, total food intake, glycated haemoglobin, total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol and triglyceride levels, and a repeated measures analysis

looked to see if there were any interactions of diet x time. All analyses were performed using Genstat statistical software (GenStat, 2003).

6.3 RESULTS

Two rats (both in the honey treatment group) had noticeable quantities of blood in their urine from day 32 onwards. These two rats were segregated from the other animals; but as they appeared to be otherwise healthy and eating well, they were not excluded from the trial. Urine samples were collected on day 40 from both animals and, at completion of the study, both animals were taken to Vet Pathology at MedLab, Hamilton, New Zealand for a full postmortem. No particular cause could be found to explain the cause of the bleeding, other than the presence of chronic cystitis in both rats. No infections were present in the urine or bladder, and it was concluded that the blood in the urine was likely to be incidental and not related to treatment. For this reason the data pertaining to these two animals was included in the statistical analyses. Data and statistical findings are given in Table 6.2.

The main results of this study are presented in Table 6.2. Overall percentage weight gain in honey-fed rats was similar to that observed in rats fed a sugar-free diet, and was significantly reduced by 16.7% ($p < 0.01$) compared with sucrose-fed rats after 6 weeks (see Figure 6.1). A repeated measures analysis of the weight data further suggested an effect of diet, with significant differences observed between rats fed sucrose and those fed either the sugar free or honey diet (both $p < 0.001$). In addition, the differences in weight between animals fed the mixed sugars and those fed honey approached significance ($p = 0.06$). Figure 6.2 shows the mean percentage weekly weight gains for all four treatments.

Total food ($p < 0.01$) and calorie intake ($p < 0.001$) were significantly higher in the sucrose, mixed sugars and honey treatments compared with the sugar-free treatment group. Furthermore, calorie intake was approximately 5% lower in honey-fed animals compared with those given sucrose ($p < 0.01$).

HbA1c levels were significantly lower in rats fed the sugar-free diet (4.02 mmol/l) than in rats fed the other three diets (4.09–4.14 mmol/l; all $p < 0.05$), but there were no differences observed between the other three diets. In addition, no differences in total-, LDL- and HDL-cholesterol or triglyceride levels were observed after 6 weeks of feeding between any of the respective diets. However it must be noted that due to difficulties with the cardiac puncture method, we were unable to collect sufficient blood from analyse 10 replicates for each treatment.

As well the variation for all cholesterol parameters was high for all treatments, and this may have masked any significant differences that may have been present. It is unknown whether this variability is due to the method of collection, the inherent inter-animal variation, or the assay employed.

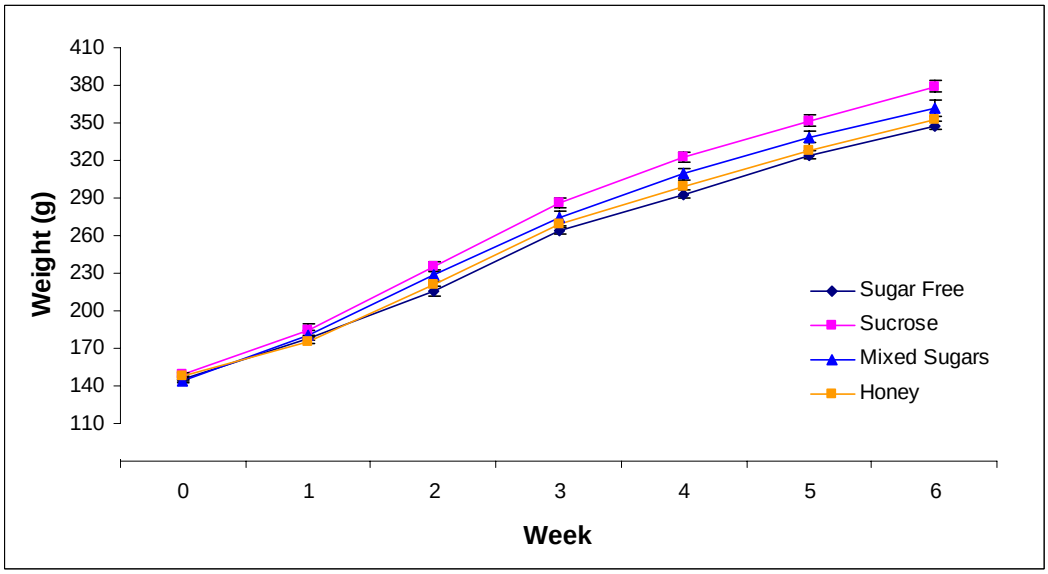

Figure 6.1: *Actual weight gains (mean ± SEM) for rats fed diets containing either no sugar (diet #1), 8% sucrose (diet #2), 8% mixed sugars (diet #3) or 10% honey (diet #4). Diets # 1, 2 and 3 had 20 ml of water added per kg of diet such that the moisture contents were the same in all three diets.*

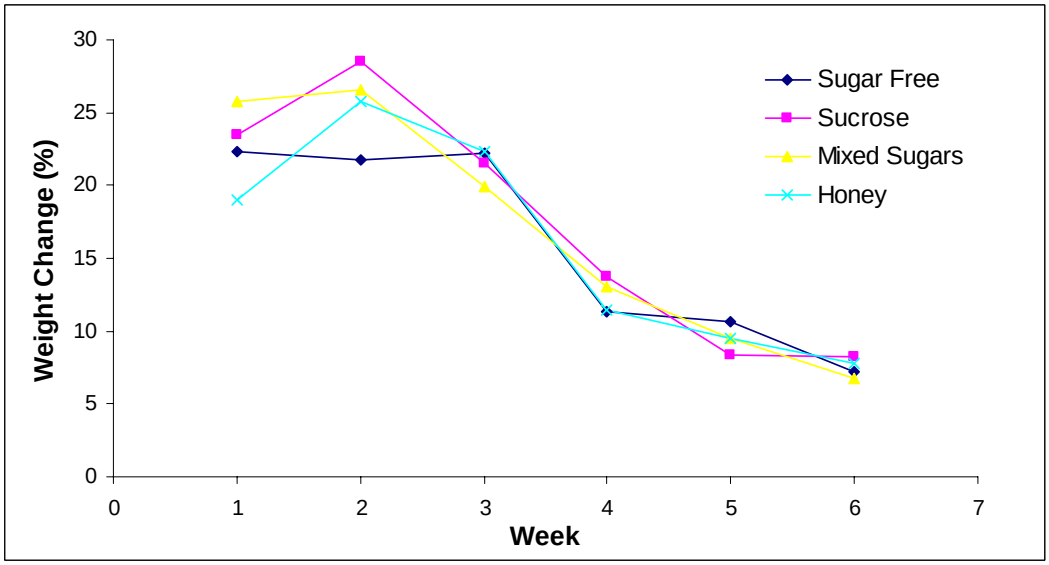

Figure 6.2: *Mean percentage weekly weight gains for rats fed diets containing either no sugar (diet #1), 8% sucrose (diet #2), 8% mixed sugars (diet #3) or 10% honey (diet #4). Diets # 1, 2 and 3 had 20mL of water added per kg of diet such that the moisture contents were the same in all three diets.*

Table 6.2: Endpoint measurements for rats fed the experimental diets for 6 weeks in a weight-gain evaluation study.

Result	Unit	Diet			
		Sugar-free (Diet # 1)	Sucrose (Diet # 2)	Mixed Sugars (Diet # 3)	Honey (Diet # 4)
Overall weight gain	%	139.7 ± 8.6†	155.0 ± 6.5**	151.5 ± 14.4	138.3 ± 11.2†
Overall food intake[a,b]	g/6 weeks	1643 ± 62	1824 ± 63**	1762 ± 47*	1772 ± 77*
Calories consumed	kJ/6 weeks[c]	29600 ± 1116	33214 ± 1144***	33010 ± 874***	31565 ± 1376***†
HbA1c[c]	mmol/l	4.02 ± 0.12	4.14 ± 0.07*	4.09 ± 0.11*	4.11 ± 0.19*
Total Cholesterol[c]	mmol/l	2.83 ± 0.62	2.69 ± 0.59	2.34 ± 0.36	2.66 ± 0.34
LDL Cholesterol[c]	mmol/l	0.31 ± 0.18	0.2 ± 0.16	0.09 ± 0.09	0.18 ± 0.18
HDL Cholesterol[c]	mmol/l	2.09 ± 0.46	2.1 ± 0.43	1.93 ± 0.3	2.07 ± 0.18
Triglycerides[c]	mmol/l	0.93 ± 0.30	0.9 ± 0.24	0.74 ± 0.38	0.83 ± 0.28
Total body fat	%	7.8 ± 1.2	8.8 ± 3.5	9.5 ± 3.6	8.8 ± 2.8[d]
Total Protein	%	20.9 ± 0.6	20.9 ± 1.3	20.6 ± 1.0	20.5 ± 41.0[d]

* p< 0.05, ** p < 0.01, *** p < 0.0001 *vs* sugar-free diet; † p < 0.01 *vs* sucrose diet
HbA1c = glycated haemoglobin; HDL = high density lipoprotein; LDL = low density lipoprotein
[a] Food intake is assessed per cage (i.e. per 2 animals). As such, there are only 5 units per treatment.
[b] Food intake corrected for percentage dry matter (determined by heating duplicate samples for 16 hours at 105^{o}C)
[c] Due to an inability to collect sufficient blood from all animals, n=9, 10, 7 and 8 for rats fed sugars free, sucrose, mixed sugars and honey diets, respectively.
[d] n=8 only, as two animals from this treatment group had blood in their urine and were investigated by Vet Pathology

Total body fat levels also varied considerably, coefficients of variation being 31.5–39.4% for the animals fed the sucrose, mixed sugars or honey. As such, no significant differences were observed between treatments, and the data numerically suggests that the percentage body fat levels were the same in honey-fed rats as in the sucrose-fed rats (both 8.8%) despite the significant difference in weight gain. Although possible, it is unlikely that honey- and sucrose-fed rats had the same levels of body fat with a >15% difference in overall percentage weight gain. Although the samples were well mixed prior to sampling, the animals were not skinned prior to mincing, and the testing laboratory noted that because the ground rat samples contained large amounts of skin and hair, it was hard to get a representative sample from each animal (sampling size was only 1 g).

To determine whether the samples were not ground sufficiently, two samples were ground further at the Massey University laboratory before assaying again with a larger sample size (3.5 g). As the data in Table 6.3 shows, there was little change in mean values compared with the original data.

Table 6.3: *Percentage body fat levels, as measured using Soxhlet extraction, after single (original) and double (repeat) mincing sessions. Sampling size for assay were 1 g and 3.5 g, respectively.*

	Sample					
	#36 rep 1	**#36 rep 2**	**Mean**	**#37 rep 1**	**#37 rep 2**	**Mean**
Original	6.65	8.57	7.61	6.55	7.33	6.94
Repeat	7.15	8.39	7.77	7.27	6.94	7.11

It is possible that the variation in these body fat data may be a result of the inability to sample accurately. The average weight of the rats at completion of the study was 350 g, therefore a 3.5 g sampling represents 1%. Differences in the amount of skin/hair and animal tissue that were collected in each sample may have dramatically altered the amount of body fat in each sample. An alternative method of body fat measurement (DEXA) will be used in future studies where body fat measurements are required.

Total percent protein levels were comparable for all four treatments.

6.4 DISCUSSION

As the results of this study demonstrate, the type of sugars that are ingested as a part of the daily diet can have a significant impact on levels of food intake and weight gain during *ad libitum* feeding. Overall percentage weight gain, for example, was significantly higher in rats fed the mixed sugars- and sucrose-containing diets compared with those fed the sugar-free diet. This may be accounted for by the increased kilojoule intake, although research is equivocal. Kanarek and Orthen-Gambill (1982) demonstrated that animals given access to 32% solutions of sucrose or fructose in addition to their standard diet gained more weight per calorie consumed than animals given only the standard (sugar-free) diet after 50 days. In addition, a number of other researchers have reported that weight gains in rodents are still higher with sucrose even when matched with caloric-equivalent starch diets (Reiser and Hallfrisch 1977; Hallfrisch and Reiser, 1981; Hill *et al.*, 1980; Morris *et al.*, 2003).

Importantly, the weight gain observed with the mixed sugar-fed rats did not occur in the honey-fed rats even though both treatments had similar food/energy intakes. Instead, weight gain was comparable between rats fed honey and the sugar-free diet even though food intake was significantly higher in honey-fed rats. As discussed previously in Chapter 5, limited research has demonstrated that honey can have a laxative effect *in vivo* (Ladas *et al.*, 1995, Ladas and Raptis 1999) due to its high fructose content. It has been suggested that the sugar composition of honey could lead to increased gut transit and/or decreased absorption and that this may account for why reduced weight gain was observed in the presence of increased kilojoule intake. However, the finding in this trial that only honey-fed rats and not those fed mixed sugars (where the composition was the same as the sugars in the honey), had reduced weight gain/food intake would suggest that the difference in weight gain is not due solely to the sugar components of the diet.

These differing results between rats fed honey and those fed mixed sugars does agree with the ANCOVA results obtained in Chapter 5. However, whereas the statistical difference in Chapter 5 was observed because of a numerical reduction in food intake in animals fed the mixed-sugars diet (mean food intake was 1263 *vs,* 1323 g) this slight difference in food intake was not seen in the present study (mean food intake 1762 and 1772 g). Unfortunately, because of the fact that the rats in the current study were housed two animals to a cage, an ANCOVA analysis using food intake as a co-variable could not be performed. Thus, it is impossible to know whether the difference in weight gain

between honey- and mixed-sugars-fed rats in this study is also due to differences in food intake. When taken together, the results of these two chapters suggest that the differences in weight gain seen in honey-fed rats may be due, in part, to non-sugar components. Indeed, when looking at the food intake data for this study, it appears that all of sweetened diets (*i.e*. sucrose, mixed sugars and honey) resulted in a higher food intake compared with the sugar-free diet. This suggests that improved palatability may account for the increased weight gain seen, with something present in the honey diet preventing absorption of some of the sugars. What these molecules could be is unknown at present, but it could be that certain molecules are interacting with the absorption of food from the gut. As data from Chapter 4 showed, a high honey diet can reduce protein absorption and increase the faecal excretion of nitrogen. A number of hypotheses were presented in Section 4.4 as to what may be going on, including the presence of protease inhibitors and/or tannins preventing protein digestion, although the discussion was speculative at best. Alternatively, the antioxidant content of the honey could increase thermogenesis of brown adipose tissue as seen in rats fed a green-tea containing diet (Choo *et al*., 2003), and this, in turn, could result in less weight gain. Further studies are needed to evaluate these hypotheses.

Unfortunately the measure of percentage body fat was inconclusive in this trial due to the large degree of variation within each treatment group, although the percentage protein levels were similar for all three treatments, indicating that consumption of honey did not lead to a reduction in protein absorption. This then would suggest that the difference in weight gain seen in this study was due to either fat, bone or water. It could be that the effect of honey on protein absorption seen in Chapter 4 was dose-related, and that honey may influence absorption in other ways when eaten in lower quantities. The faeces were not collected in this study, and we have no measure of whether there was increased total excretion, or altered excretion of either fat, sugar or protein, but it would be worth assessing these measures in future studies.

It must be noted here, though, that despite the fact that comparisons are being drawn between the data in this study and that obtained in Chapter 5, the diet compositions differed between these two studies. The diets used in Chapter 5 were based on those used in other rodent weight assessment studies done at Crop and Food Research whereas the study reported here was formulated to simulate a typical New Zealand diet. However both diets provided levels of fat, protein and sugars well in excess of those required for the maintenance or growth in rodents. In addition, the animals were different ages in the two studies (animals

were aged 14 weeks at the start of the study in Chapter 5, and 6 weeks at the start of the present study). In other studies, adult rats given sucrose displayed increased body weight after 1–2 weeks (Kanarek and Hirsch 1977; Kratz and Levitsky, 1979) whereas weanling rats given sucrose consumed more calories than their sugar-free counterparts, but little measurable differences in weight gain/kilojoule were observed until they reached 10–12 weeks of age (Kanarek and Marks-Kaufmann, 1979). Similar data for combinations of fructose and glucose (as in honey) in the gut of weanling and older animals is not available, but it is possible that absorption of these sugars may have been more pronounced in adult animals and/or that little sugar absorption occurred in younger animals. Furthermore, both weight studies used honey from the same floral source, thus it is possible that the factor(s) involved originate from the plant yielding the honeydew. It would therefore be of interest to assess the effects of honey of differing floral sources on weight gain.

Although not measured in this study, it is possible that the lower weight gains observed in honey-fed animals compared with those fed sucrose are partially due to differences in satiety or glycemic control; however, given that starch was the major component in all diets, these differences are probably minor at best. The honey-fed rats did eat numerically less than those fed sucrose, and this could account for the decrease in weight gain seen between these two groups. It could be that consuming honey rather than sucrose leads to more stable blood sugars for longer (rather than a rapid increase followed by a rapid decrease as is seen with sucrose) which, in turn, may lead to less hunger and less need/desire to eat. To ascertain whether this hypothesis were true a larger study, with a greater power would of course be needed to determine if this numerical difference in food intake was real or simply due to data variability. Another explanation of the lower weight gains seen in the honey-fed rats is the production of hydrogen peroxide by the honey, as *in vitro* research has shown hydrogen peroxide to be a strong insulin-mimetic agent (Czech *et al.*, 1974; Hayes and Lockwood 1987; Heffetz *et al.*, 1990). Levels of hydrogen peroxide production in honey vary depending on floral source, but were shown to be in the range of 0.5–3.5 mmol/l in high peroxide-producing honeys (Bang *et al.*, 2003). and these levels agree with the 1–3 mmol/l shown to induce tyrosine phosphorylation (an insulin-mimetic effect) *in vitro* (Hayes and Lockwood 1987; Heffetz *et al.*, 1990). In addition, iron-binding antioxidants in particular honeys (including that used in the current study) have been shown to reduce the destruction of hydrogen peroxide that usually occurs via the Fenton reaction

(discussed previously in Section 4.4) It is possible; therefore, that hydrogen peroxide originating from the honey acts to facilitate the uptake of glucose from the blood (by stimulating insulin receptors on the cells), thereby preventing the surge in blood glucose and insulin that usually follows consumption of a high carbohydrate meal.

Limited clinical studies have demonstrated that honey, sucrose and fructose do have differential effects on blood glucose levels (Samanta *et al.*, 1985; Al-Waili, 2003c, 2004b). In the two clinical studies (Samanta *et al.*, 1985; Al-Waili, 2004b), plasma glucose levels were significantly lower after 1–3 hours in both healthy and diabetic patients who had consumed honey (20–75 g) than in those who had consumed equivalent amounts of dextrose, sucrose or fructose. Similarly, sheep given daily IV infusions of honey (50–80 grams daily for 10–14 days) had decreased blood sugar levels compared with animals fed equivalent amounts of dextrose (Al Waili, 2003b). It is possible, therefore, that the ingestion of honey in the present study may have resulted in sustained lower plasma glucose levels, thereby resulting in reduced fat deposition. Of course, large differences in metabolism and activity occur between humans and rodents, and direct inferences cannot be drawn.

Whilst there appears to be no evidence in the literature to demonstrate that hydrogen peroxide is absorbed from the gut or that the levels produced in honey are sufficient to trigger insulin receptors, the findings from this study do demonstrate that intake of honey can lead to reduced levels of weight gain. In particular, the fact that honey but not mixed sugars has a lower weight gain, yet similar food intake, compared with the sucrose diet suggest that there is something present in the non-sugar component of honey that results in decreased weight gain per kilojoule of food consumed.

Other rodent studies have also demonstrated that animals fed a high fat, high sugar diet display glucose intolerance, insulin resistance and reduced adipose tissue lipoprotein lipase activity when compared with animals fed standard chow (Stark *et al.*, 2000; Picard *et al.*, 2002). It is unknown whether the rats in this study exhibited these effects also, but it would appear likely given the high fat/high sugar diet composition. It is possible, therefore, that the effects of honey may have been mediated by an altered glycemic response due to insulin resistance, and that this may not have occurred or been observed if the dietary makeup had been different. The finding is still significant, though, as several clinical studies have demonstrated that glucose intolerance and insulin resistance also occur in humans consuming a high fat/high sugar diet (Eckel *et al.*, 1995;

Ong and Kern, 1989). Furthermore, the diets that these rats were fed was specifically designed to match the typical New Zealand diet, so that any such effects of diet on blood sugars responses would occur in a similar fashion.

It is also possible that the honey may have directly increased the basal metabolic rates (BMR) of these animals, or resulted in increased energy levels leading to higher energy expenditure. This is questionable, though, as all animals were housed in relatively small cages (45 x 25 x 30cm high) that did not allow them a lot of room to play or exercise. However, rats are nocturnal animals so would have been more active at night. None of these animals were observed between the hours of 6pm and 8am so it is impossible to say that activity levels were not increased in honey-fed animals.

Other research on humans has also demonstrated that honey can have beneficial effects on lipid profiles. Al-Waili (2004b) reported that whereas a single dose (75 g/250 ml) of dextrose or artificial honey led to increases in cholesterol and triglycerides over 1–3 hours, the effect was not observed with honey. In this same study, daily feeding of honey for 15 days decreased cholesterol by 8%, LDL-cholesterol by 11% and C-reactive protein by 57% in patients with elevated cholesterol compared with baseline values. No effects on total cholesterol, LDL-cholesterol, HDL- cholesterol or triglyceride levels were observed in the present study with any of the four treatments, however, due to difficulties with blood sampling we were unable to collect sufficient blood from all animals to allow 10 replicates to be analysed for each treatment. As well, the variation for all cholesterol parameters was high for all treatments, and this may have masked any significant differences that may have been present.

Housing animals in pairs is not ideal for weight investigation studies, and this may have affected the results obtained. O'Connor (2000) has demonstrated that food intake in rats is higher when animals are housed in pairs compared with when they are housed individually. When housed in pairs one animal may dominate over the other and consume more food. Fighting was common in all the cages, particularly as they got bigger, and this may have been to exert dominance within the cage. On certain weigh-in dates, particular cages did show a discrepancy between the weights of the two animals (despite similar weights the week before) suggesting that one animal may be dominating the access to, or intake of, food. Approximately 60 g of food was provided per cage daily, and this is well in excess of that consumed by the two animals. However, at certain times the food container was dislodged and tipped up such that the food spilled through to the blotter beneath the mesh. As well, some rats were messy eaters, digging at

the diet with their front paws, spilling diet in the process. It is possible; therefore, than one of the rats in a given cage may have had less access to food due to the other animal inadvertently spilling too much such there was insufficient left for the other animal. However, a look at the complete 6-week weight data did not show any patterns for reduced weight gain for a given animal nor for ongoing weight discrepancies for any of the cages.

Although honey does appear to have a significant effect on weight gain in weaning rats (and potentially in adult rats also), we cannot automatically assume that the effect will be the same in humans. Ong and Kern (1989) and Swierczynski *et al.*, (2000) have demonstrated that the activities of lipogenic enzymes are comparable in rodents and human when the macronutrient compositions are the same (as in this study) but mass, physiology, activity level, stress levels differ significantly between these two groups (the use of rat models to assess human health is discussed in Chapter 11). In this study, for example, animals were given access to only powdered diet and water. In the real world, people have ready access to a huge variety of foods, ranging from the healthy, nutritious fruit and vegetables to the less desirable high fat, highly processed snacks. It is fairly unlikely that many people would replace all the sugar in their diet with honey or that they would consume enough honey to have a significant effect on their weight. Weight loss depends on controlling calorie intake as well as calorie expenditure and weight loss is not likely to occur in an individual that consumes significant quantities of honey as well as excess calories from other foods. However, food producers may in time take some responsibility for the "healthiness" of the foods they produce and supply. The replacement of sugars (including sucrose and fructose) with honey in particular foods and beverages may provide sweetness without additional calorie-induced weight gain. A trial in humans instead of in rats would be needed to determine if such a benefit to health would result.

Chapter Seven:

The Effect of Honey versus Sucrose on the Recovery of Bone Density Following a Calcium-Deficient Diet in Rats

This chapter presents the information from the first of two rat-feeding trials that were designed to assess the effect of honey on calcium uptake (the second trial is presented in Chapter 8). This trial was carried out at Crop and Food Research (CFR) in Palmerston North in 2004, and was fully funded by a CFR EUREKA award. As with the weight regulation study funded by CFR (see Chapter 5), funding was limited. Thus the experimental design and analyses undertaken were chosen to fit within budgetary constraints.

A brief discussion of the results of the trial presented in this chapter will be given in Section 7.4, and a more detailed discussion of the roles of sugar and honey on calcium absorption and bioavailability will be given at the end of Chapter 8.

7.1 INTRODUCTION

Calcium is an essential dietary nutrient, and both scientists and the general public are now becoming aware of the importance of consuming

sufficient calcium. The recommended daily intake (RDI) of calcium is about 900 mg/day (800–1000 mg/day depending on the country) for adults although levels as high as 1300 mg/day are recommended for adolescents, pregnant women and the elderly (Gueguen and Pointillart, 2000). Various studies are now showing that many individuals are often consuming less than two thirds of the RDI (Heseker *et al*., 1992; van Dokkum, 1995; Gueguen, 1996). Those that choose to exclude dairy products from their diet are particularly at risk as approximately 70% of dietary calcium comes from milk and other dairy products (although minimal levels are also provided in green vegetables, fruits and water) (Gueguen and Pointillart, 2000).

Calcium has numerous roles in the body including regulation of enzyme activity, blood clotting, secondary messenger systems in the cell and the hydrolysis of energy rich phosphate bonds in ATP to fuel work (Wood, 2000). However, approximately 99% of the body's calcium (about 1200 g in total) is involved in the formation of bone and teeth (Wood, 2000). Bone calcium stores act as a reservoir of calcium for the body, their levels getting depleted to maintain levels for other functions of calcium if intake is not sufficient (Wood, 2000). Reductions in calcium intake are therefore most often reflected in decreased bone strength and quality. Osteoporosis is a disease that is associated with a life-time of inadequate calcium intake (although other factors including vitamin D and hormonal levels are also involved). It is characterised by a reduction in bone mineral density, disrupted bone microarchitecture and alterations in the amount and variety of non-collagenous proteins in bone (Sowers, 1996), and affects an estimated 200 million people worldwide (Gueguen and Pointillart, 2000). While treatment options such as the bisphosphonates are becoming available, prevention is still considered the most important way to reduce the incidence and severity of osteoporosis. As well, research is now showing a link between reduced dietary calcium intake and other diseases including arterial hypertension and colon cancer (Sowers, 1996). The ability to increase calcium absorption is therefore of both nutritional and economical importance, and research is now focussed on determining how calcium absorption and bioavailability can be improved, and whether calcium supplementation can retard the rate of bone loss and calcium-related disease formation in older, at-risk individuals.

Calcium absorption occurs via two mechanisms, depending on its location in the gut. Active transport (an energy-dependent process) occurs mainly in the duodenum and upper jejunum, whilst passive diffusion occurs throughout the small intestine. Calcium is also significantly absorbed from the large intestine,

particularly the caecum and the colon (Younes *et al.,* 2001). For calcium to be able to cross the intestinal wall, it must be in a soluble form (generally ionised Ca^{2+}) or bound to a soluble organic molecule (Gueguen and Pointillart, 2000). However, calcium is readily bound to phosphate and other ions, resulting in the rapid formation of insoluble calcium complexes. As a result, less than 50% of consumed dietary calcium is actually absorbed (Younes *et al.,* 2001).

Passive diffusion, unlike active transport, is a unsaturable process that can increase with dietary intake (Bronner, 1992). Thus, substances that can make calcium soluble or keep calcium soluble for longer should stimulate passive diffusion, thereby increasing calcium absorption. Casein phosphopeptides, for example, form soluble chelates with calcium (Bronner, 1987) and their ability to increase calcium absorption *in vivo* is well established in the literature (readers are referred to the reviews by Meisel, 1997; Clare and Swaisgood, 2000; Scholz-Ahrens and Schrezenmeir, 2000). Limited research has demonstrated that sugars alcohols such as xylitol, erythritol, sorbitol, maltitol and lactitol can increase calcium absorption from both the large and small rat intestine (Hamalainen and Makinen, 1986; Hamalainen *et al*, 1990; Abrams *et al*., 2002; Mineo *et al*., 2002) and one theory is that this is due to the complex formation between polyols and calcium in the intestinal tract (Hamalainen and Makinen, 1989). However, results from clinical studies have not supported these data (Zitterman *et al*., 2000).

Similarly, carbohydrates that are more slowly absorbed have also been shown to improve calcium absorption. These “fermentable carbohydrates” as they are often known, reach the large intestine largely undigested where they are fermented and utilised by the local microflora. This may stimulate gut bifidobacteria and lactobacilli growth in the intestine, which has been suggested to have health promoting functions (Gibson *et al*., 1995; Kruse *et al.,* 1999). Importantly, bacterial carbohydrate fermentation can result in the formation of short chain fatty acid (SCFA) fermentation products. These SFCA can reduce the pH of the caecal content (which increases mineral solubility and mineral absorption) and affect absorption directly by complexing with the calcium, resulting in improved absorption (Younes *et al.,* 2001). Animal studies have demonstrated that rats absorb more calcium and have higher levels of bone recalcification than control rats if fed fermentable carbohydrates such as inulin (Niness, 1999) and resistant starches (Ohta *et al.,* 1994, 1995, 1996; Delzenne *et al.*, 1995, Younes *et al*., 2001). Similarly, Brommage *et al*. (1993) and Beynen *et al*. (2001) demonstrated that lactulose (a disaccharide of galactose and fructose)

is also rapidly fermented by gut bacteria leading to increased calcium absorption in dogs. In addition, inulin has also been shown to increase calcium uptake in a small number of clinical studies (Coudray *et al.,* 1997; Coudray and Fairweather-Tait, 1998, Van den Heuvel *et al*., 1998).

Honey is approximately 80% sugars by weight, including both simple and complex sugars. As well it contains other components including the enzyme glucose oxidase that produces gluconic acid from glucose and oxygen (see Chapter 1). As research has previously demonstrated that honey exhibits reduced glucose absorption in the gut because of its high fructose content (Ladas *et al.,* 1995; Ladas and Raptis, 1999), and that the presence of glucose in the gut can stimulate calcium absorption directly (Younoszai and Nathan, 1985; Wood *et al*., 1987) it is likely that honey may also have a positive effect on calcium absorption *in vivo*. Indeed, in one small clinical study carried out in the early 1940s (Knott *et al*., 1941) calcium retention was shown to be higher in babies fed formula containing honey than in those fed standard formula alone. The current trial was therefore designed to determine if feeding honey to calcium-deficient rats could improve calcium absorption and bone calcium levels. The basic study design is based upon that of Hamalainen *et al.* (1990) and Hamalainen (1994).

7.2 MATERIALS AND METHODS

7.2.1 Experimental Animals

Weaned, Sprague Dawley male rats (21-23 days, 45-50 g) were used in the feeding trial and were bred at the Food Evaluation Unit, Crop & Food Research, Palmerston North, New Zealand. The trial was carried out in the Food Evaluation Unit as described in Section 2.1.1 with full ethical approval (see Section 2.1.4). These animals were bred specifically for this study, and were housed in groups with access to rodent pellets prior to commencing the study.

7.2.2 Experimental Diets

A honeydew honey (from *Nothofagus solandrii*) with a known rate of hydrogen peroxide production (antibacterial activity due to hydrogen peroxide equivalent to that of 18.6% phenol; Allen *et al.,* 1991) and a high antioxidant content (TEAC = 2.9 mmol/l) was supplied by the Honey Research Unit, Waikato University, New Zealand for this study. The water content of the honey was determined using a refractometer and shown to be 20%.

A calcium-free, sucrose-based diet (diet #1027) and two calcium-rich diets containing either 8% sucrose (diet #1028) or 10% honey (diet #1029) were

prepared by Crop and Food Research and stored at 4°C (see Table 7.1 for diet composition). A standard vitamin mix and a modified calcium-free salt mix were prepared in-house at CFR, the details of which are given in Chapter 2. Where required, calcium (in the form of $CaHPO_4$) was added to the diet at a level of 20.7 g/kg (providing 6 g calcium per kg diet).Twenty millilitres of water was added to each kilogram of sucrose diet to account for the fact that the honey contained 20% water. Sub-samples (~10 g) were taken from each diet at the start of each week and dry matter contents calculated by drying for 16 hours overnight at 105°C.

Table 7.1: *Composition of Experimental Diets (g/kg)*[a]

Ingredient	Ca-deficient diet (Diet #1027)	Sucrose diet (Diet #1028)	Honey diet (Diet #1029)
Lactalbumin	120	120	120
Corn Oil	65	65	65
Sucrose	80	80	0
Honey	0	0	100
Cellulose	50	50	50
Ca-free Mineral Mix	50	50	50
$CaHPO_4$	0	20.7	20.7
Vitamin Mix	50	50	50
Starch	585	564.3	564.3
Water	20 ml	20 ml	0

[a] Supplier information for all ingredients is given in Chapter 2.

7.2.3 Experimental Procedures

Thirty weanling rats were placed into raised stainless steel cages with 6 mm^2 grid floors. They were randomly allocated to receive either the standard calcium-containing sucrose diet (diet 1027; treatment groups 1 and 2; n = 6 per group) or the calcium-deficient sucrose diet (diet 1028; treatment groups 3, 4 and 5; n = 6 per group) *ad libitum* for 21 days (Phase 1). Water was available to the rats at all times. Live weights were recorded for each rat every seven days, and food intakes were calculated for every seven-day period.

On day 19, the 12 animals from treatment groups 2 and 3 were placed into clean metabolism cages and their urine and faeces collected during a 48

hour period. Excretions were discarded from the first 24 hour period, with total urine collected during the second 24 hour period and frozen at –80^{o}C for later analysis of urinary calcium. On day 21, these 12 animals were anaesthetised using CO_2 gas, and 3–6 ml of blood removed via cardiac puncture (using 18 gauge needles). The rats were then fully euthanised with CO_2 and the bodies placed in numbered bags for dissection. The blood samples were left to clot at room temperature before centrifuging at 3000 x *g* for 10 minutes. The serum was removed, transferred to clean, labeled Ependorf tubes and stored at –80^{o}C for later analysis of plasma calcium levels.

From day 21 onwards, the calcium-deprived animals in treatment groups 4 and 5 were fed calcium-containing diets that contained either sucrose (diet #1028), or honey (diet #1029) for a further 21 days (Phase 2). Rats in treatment group 1 continued to receive the standard calcium-sucrose diet during this time. A diagrammatic representation of the dietary treatments for Phase 1 and 2 is given in Figure 7.1. Live weights and food intakes were again recorded for each rat for every seven-day period.

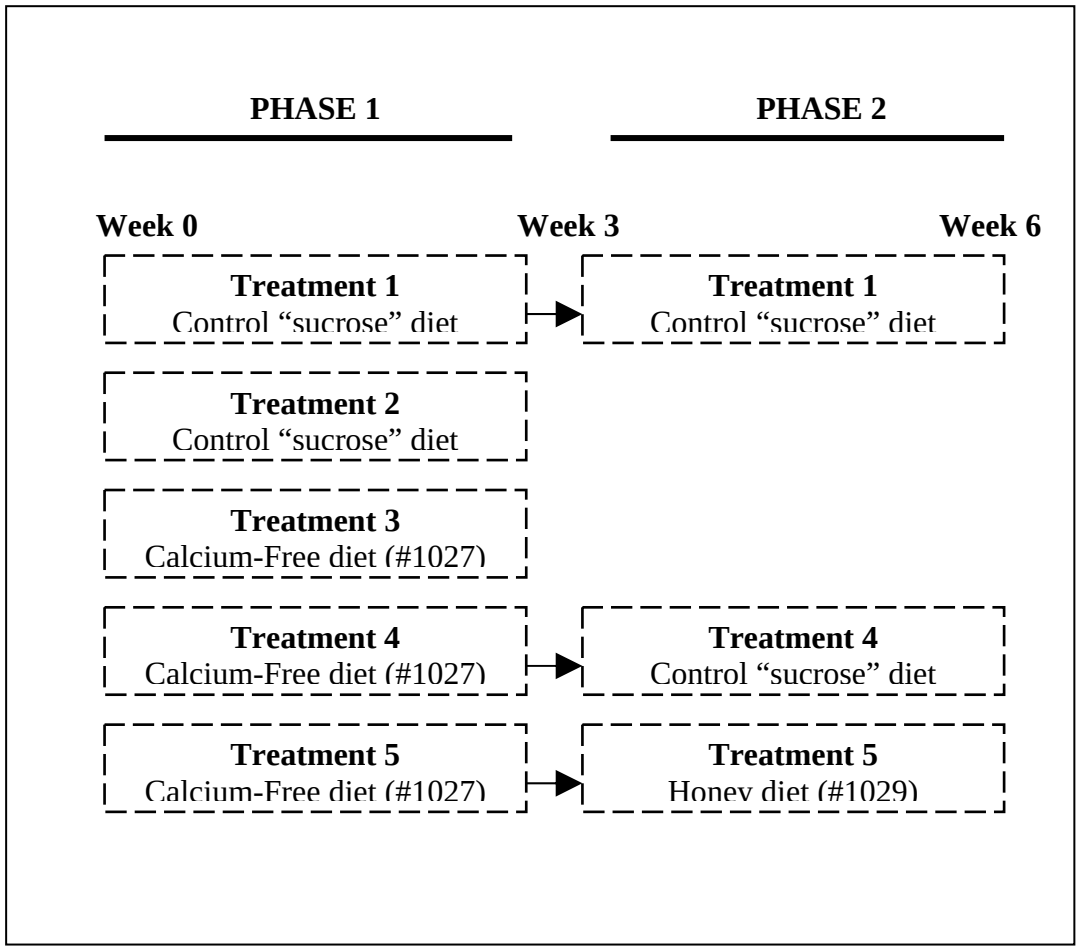

Figure 7.1: *Schematic representation of the dietary treatment regime*

On day 40, all remaining animals were transferred to clean metabolism cages. Urine and blood samples was collected and processed on day 42 as described previously, and the animals bagged for dissection.

Left fore and hind limbs were sectioned from each animal. The humerus and the fibula/tibia were carefully removed and the attached soft tissue removed. The bones were then left to air-dry at room temperature for 72 hours. After this time, all remaining soft tissue material was carefully removed from each bone using a scalpel. The individual bones were weighed, and their volumes recorded by placing into a 5 ml measuring cylinder that was two thirds filled with distilled water and measuring the increase in volume. All urine, blood and bone samples were then sent to the Nutrition Laboratory at Massey University for measurement of calcium levels (see Chapter 2 for details).

7.2.4 Statistical Analyses

One-way ANOVA analyses were carried out on weight gain, food intake, bone weight and volume as well as on bone, serum and urinary calcium levels to determine whether there were statistically significant differences between the calcium levels of control and calcium-deprived rats, and/or between calcium-deprived rats fed the sucrose- and honey-based diets. All analyses were performed using Genstat statistical software (GenStat 2003).

7.3 RESULTS

7.3.1 Phase 1 (Weeks 1– 3): Assessment of Feeding a Calcium-Free Diet *vs* the Control Sucrose Diet

7.3.1.1 Weight Gain/Food Intake

Animal weights were significantly reduced by approximately 25 g in rats fed the calcium-deficient diet (Treatments 3, 4 and 5) compared with those fed the control calcium-containing sucrose diet (Treatments 1 and 2) after 3 weeks of treatment ($p < 0.001$; Table 7.2). Similarly, total food intake during weeks 1–3 was significantly reduced in rats fed the calcium-deficient diet compared with animals fed the control ($p < 0.001$; Table 7.2).

Table 7.2: *Assessment of Phase 1 (Weeks 1–3) weight gain and food intake (mean ± SD) for rats fed either the control sucrose-based diet (Treatments 1 and 2) or the calcium-deficient diet (Treatments 3, 4 and 5). W=week*

Parameter	Calcium-containing sucrose Diet (#1028)	Calcium-Free Diet (#1027)
Live weight at W3 (g)	152.2 ± 6.7	126.8 ± 9.2*****
Δ Live weight (g, W3–W0)	99.5 ± 7.0	75.1 ± 9.0 *****
Food Intake (g) [a]	275.3 ± 17.5	233.5 ± 15.8*****

***** $p < 0.001$ vs calcium-sucrose (#1028) diet
[a] Food intake corrected for percentage dry matter

7.3.1.2 Bone Data and Serum/Urinary calcium levels

The bone, serum and urinary calcium data obtained from animals sacrificed at Week 3 (treatments 2 and 3) are given in Table 7.3. The mean calcium content of both fibula/tibia and humerus bones were significantly reduced by approximately 50% in the calcium-deprived rats compared with the control rats fed the calcium-sucrose diet at Week 3. Mean bone weight and mean bone densities were significantly reduced by 35–40% and 25%, respectively, in the calcium-deprived rats (all $p < 0.001$ vs the control diet). In addition, mean serum and urinary calcium levels were reduced by 30% and 60%, respectively, in the calcium-deprived rats compared with control animals (both $p < 0.001$).

Table 7.3: *Assessment of Week 3 bone measurements and urinary/serum calcium levels (mean ± SD) for rats fed the either the control sucrose-based diet (Treatments 1 and 2) or the calcium-free diet (Treatments 3, 4 and 5).*

Parameter	Calcium-containing Sucrose Diet (#1028)	Calcium-Deficient Diet (#1027)
Fibula/Tibia		
Calcium content (mg/g)	116.8 ± 30.1	56.3 ± 18.3*****
Bone Volume (ml)	0.33 ± 0.05	0.28 ± 0.03
Bone Weight (mg)	0.25 ± 0.02	0.16 ± 0.01*****
Bone Density (mg/ml)	0.76 ± 0.07	0.58 ± 0.08*****
Humerus		
Calcium content (mg/g)	129.2 ± 8.6	72.6 ± 10.6*****
Bone Volume (ml)	0.16 ± 0.02	0.13 ± 0.03
Bone Weight (mg)	0.13 ± 0.01	0.08 ± 0.01*****
Bone Density (mg/ml)	0.82 ± 0.09	0.62 ± 0.09*****
Urinary calcium (mmol/l)	20.7 ± 5.8	8.8 ± 3.3*****
Serum calcium (mmol/l)	3.0 ± 0.1	2.1 ± 0.3*****

***** $p < 0.001$ vs control (#1028) diet

7.3.2 Phase 2 (Weeks 4–6): Assessment of Feeding a Calcium-Containing Sucrose- or Honey-Based Diet to Calcium-Deficient Rats *vs* Control Animals.

7.3.2.1 Weight Gain/Food Intake

Animal weights at Week 6 remained significantly lower in animals fed the calcium-deficient diet during Phase 1 (Treatments 4 and 5) when compared with control animals (Treatment 1) ($p < 0.05$). However, the weight gain per 3 weeks was not significantly different in the three treatment groups (see Table 7.4). In addition, no significant differences in food intake were observed in rats fed the control, sucrose- or honey-based diets (Table 7.4).

Table 7.4: *Assessment of weight gain and food intake (mean ± SD) during Weeks 4–6 (Phase 2) for control rats continuing to be fed the control calcium/ sucrose-based diet (Treatments 1), and for calcium-deficient rats fed the sucrose- (Treatment 4) or honey-based (Treatment 5) diets. W=week.*

Parameter	Sucrose Diet (Treatment 1)	Sucrose Diet[a] (Treatment 4)	Honey Diet[a] (Treatment 5)
Live weight (W6)	287.3 ± 6.4	272.2 ± 9.0*	273.2 ± 9.4*****
Δ Live weight (W6–W3)	133.5 ± 8.1	141.9 ± 14.5	146.3 ± 12.9
Food Intake[b] (W4+W5+W6)	446.4 ± 36.4	421.0 ± 29.4	437.7 ± 34.2

[a] Rats were previously fed the calcium-deficient diet (#1027) during Weeks 1–3.

***** $p < 0.05$ vs control sucrose diet (treatment 1)

[b] Food intake corrected for percentage dry matter

7.3.2.2 Bone Data and Serum/Urinary Calcium Levels

The data obtained from animals sacrificed at Week 6 (Treatments 1, 4 and 5) are given in Table 7.5. Generally, no differences were observed in all three treatments for either bone calcium content, volume, weight or serum calcium levels and urinary calcium excretion, suggesting that the calcium stores lost in Treatment 4 and 5 rats during Phase 1 had been replenished during Phase 2 feeding. However, bone density remained lower in rats fed sucrose during Phase 2 (Treatment 4) compared with control animals (Treatment 1; $p < 0.05$) suggesting that bone density in these animals had not been fully restored. In contrast, bone density levels were fully restored in honey-fed rats at week 6..

Table 7.5: *Assessment of Week 6 bone measurements and urinary/serum calcium levels (mean ± SD) for rats continuing to be fed the control sucrose-based diet (Treatment 1), and for calcium deficient rats fed the sucrose- (Treatment 4) or honey-based (Treatment 5) diets.*

Parameter	Sucrose Diet (Treatment 1)	Sucrose Diet[a] (Treatment 4)	Honey Diet[a] (Treatment 5)
Fibula/Tibia			
Calcium content (mg/g)	120.2 ± 53.1	120.4 ± 19.0	119.2 ± 29.1
Bone Volume (ml)	0.41 ± 0.04	0.38 ± 0.03	0.41 ± 0.05
Bone Weight (mg)	0.36 ± 0.03	0.30 ± 0.02	0.34 ± 0.03
Bone Density (mg/ml)	0.88 ± 0.11	0.80 ± 0.1	0.84 ± 0.07
Humerus			
Calcium content (mg/g)	178.2 ± 35.2	148.5 ± 30.4	172.6 ± 16.4
Bone Volume (ml)	0.25 ± 0.01	0.24 ± 0.02	0.23 ± 0.03
Bone Weight (mg)	0.20 ± 0.01	0.16 ± 0.02	0.16 ± 0.01
Bone Density (mg/ml)	0.81 ± 0.05	0.68 ± 0.07*****	0.73 ± 0.08
Urinary calcium (mmol/l)	17.8 ± 3.2	15.8 ± 3.7	14.9 ± 3.5
Serum calcium (mmol/l)	3.07 ± 0.1	3.1 ± 0.3	3.0 ± 0.2

***** $p < 0.05$ vs control sucrose diet (Treatment 1)
[a] Rats were previously fed the calcium-deficient diet (#1027) during Weeks 1–3

7.3.3 Week 3 *vs* Week 6

To ensure the robustness of the trial design a number of parameters were compared between the control group of rats sacrificed at Week 3 and the control group sacrificed at Week 6.

Fibula/tibia calcium levels were not significantly different in control calcium-fed rats at Week 6 (Treatment 1) versus Week 3 (Treatment 2) (120.2 *vs* 116.8 mg/g bone); however, humerus calcium levels were greater in Week 6 control animals compared with Week 3 (178.2 *vs* 129.2 mg/g; $p < 0.05$). This difference may be artefactual due to the small numbers of animals assessed at each time period, and the fact that the bones were so small that replicates could not be analysed for each sample. No differences were observed in bone density between the two control groups although both tibia/fibula and humerus bone weights and bone volumes were significantly higher in Week 6 control animals compared with Week 3 animals ($p < 0.001$). This is to be expected because of

the increased growth and size of the animals at Week 6. Furthermore, there were no differences in either urinary or serum calcium levels in the two control groups.

7.4 DISCUSSION

As the effects of honey on calcium absorption are further explored in the rat-feeding trial in Chapter 8, the discussion in this chapter will relate only to the results obtained in this trial and the methodology employed. Further discussion on the implications of this research and on other aspects of calcium absorption and bioavailability will be presented in the discussion section of Chapter 8.

As the results of this small study show, there are no significant differences in levels of bone recalcification between calcium-deficient rats fed a diet containing 8% sucrose or 10% honey (honey is ~20% water). However, this may be a result of the trial design rather than due to similarities between the two experimental treatments. The trial design used in this study was based upon the work of Hamalainen *et al.* (1990) and Hamalainen (1994) who showed that lactose, xylitol and gluconate increased bone relcalcification compared with a sugar-free diet in calcium-deficient rats. However, three main differences were employed in the present study. Firstly, the diet composition used in this study (see Table 7.1) differs from that of the two studies by Hamalainen (20% casein, 62.7% cornstarch, 5% cellulose, 7% fat/oil). The diet in the present study was formulated based on rodent dietary requirements (National Research Council, 1995) and on other rodent diets that had been prepared by Crop and Food Research previously. Whereas both the diets in this study, and that presented by Hamalainen had similar levels of starch, fat, fibre, vitamins and minerals, the two diets differed in the level and type of protein used, the presence of sugar and the form of calcium that was used.

Casein, for example, was used in both of the studies by Hamalainen; however, lactalbumin was used as the source of dietary protein in the present study to prevent the presence of casein phosphopeptides having any effect on calcium absorption. Secondly, calcium phosphate rather than calcium carbonate was used in the present study to increase the level of calcium that would potentially form hydroxyapatite thereby rendering the calcium unavailable for absorption. This was to test the hypothesis that honey may improve bone recalcification due to the gluconate content of the honey keeping the calcium soluble in the gut. Thirdly, an 8% sucrose diet, rather than a sugar-free diet was used as the control diet in this study as the typical human diet is not sugar-free, but instead often contains a large proportion of sucrose. It may be of interest,

though, to compare the effects of both the sucrose diet and honey diet in calcium-deficient rats with control rats fed a calcium-containing, sugar-free diet.

Importantly, Hamalainen *et al.* (1990) and Hamalainen (1994) used a 2-week calcium-recovery period during Phase 2 of the study, compared with the 3 week period using in the current study. This difference in trial duration may explain why different bone calcium levels were detected with different experimental diets in their research whereas bone calcium levels in the experimental groups were comparable to those in the control group at the end of the current study. Hamalainen *et al*. (1990) mention briefly that a preliminary pilot study suggested that a 2-week period does not permit the full recovery of bone density. A 3-week period was chosen for Phase 2 in the current study as it was hypothesised that bone recalcification would be slower than in the published studies because of the absence of casein and the use of calcium phosphate in the diets. Limited resources meant that a pilot study could not be performed prior to commencing this study; although the data would now suggest that this hypothesis was not supported.

Despite the fact that there were few statistically significant differences observed between the control rats (Treatment 1) and the calcium-deficient rats fed a sucrose- (Treatment 4) or honey-containing diet (Treatment 5) after 6 weeks, a number of trends were observed. Humeral calcium content, for example, was 97% of control levels in honey-fed rats, yet only 83% of control levels in sucrose-fed animals. Similarly, bone weight and bone density values were 90–95% of control values in honey-fed rats, but were only 80–91% of control values in those fed sucrose. In addition, humeral bone density levels were significantly lower in sucrose-fed rats compared with those of control animals at 6 weeks ($p < 0.05$). It is unknown whether the non-significant findings are due to random variance, or whether these numerical differences are indicative of a potential difference in the diets that simply did not reach statistical significance because of the small sample sizes used in this study and the inherent variability that occurs in animal trials. These data suggest, though, that sucrose and honey *may* have a different effect on calcium absorption and bone recalcification, although further studies are needed to determine this.

Another point of consideration is that the rats in the present study were given calcium at a rate of 6 g/kg of bodyweight during Phase 2, which is slightly higher than the 5 g/kg required to satisfy their nutritional requirements (National Research Council, 1995). It is possible that no significant differences in calcium uptake/bone recalcification were observed between animals fed the sucrose diet

and the honey diet because of a surplus of calcium available for absorption. As mentioned previously, passive transport of calcium in the small intestine is a non-saturatable process, thus any surplus calcium in an absorbable form is likely to be absorbed and incorporated into bone stores. As humans, and particularly women, generally have an insufficient intake of calcium in their diet, it may have been of more value to feed the animals in Phase 2 with diets that contained minimal levels of calcium such that differences in uptake were more visible and 100% replenishment of bone calcium levels was not obtained by Week 6. However, given the design of the trial, and the use of calcium-deficient diets in Phase 1, it was deemed to be unacceptable to use such a low level of calcium in the second phase of the trial.

It would also have been advantageous to run the trial using a larger number of animals as there was substantial variability in all the parameters measured, particularly bone calcium levels. Fibula/tibia and humerus bones from weanling rats are very small and, once ashed, there is generally insufficient sample to allow duplicate analyses for each sample. Alternatively, both fore- and hind-limbs could be removed for analysis (rather than just one of each as used in this study); although, there is obviously a financial cost associated with needing to remove, ash and analyse twice as many bones. Instead it may be more beneficial to use larger animals in the trial which should yield larger bones that are easier to analyse. Furthermore, better analytical techniques are available for measurement of bone strength. DEXA is routinely used for measurement of bone mineral density and for diagnosis of conditions such as osteoporosis. This method was not used in the present study as it was financially beyond the scope of this project. In addition, it would have been advantageous to have an additional treatment group that assessed the effects of honey on bone calcium levels in healthy animals that were not calcium-deficient (*i.e.* fed the control sucrose diet in Phase 1, followed by honey in Phase 2). These factors have been considered in the design of the next calcium trial, and the data is presented in Chapter 8.

Chapter Eight:

The Effect of Honey versus Sucrose on Calcium Bioavailability in Weanling Rats Fed a Low Calcium Diet

This chapter presents the data from the second rat-feeding study that was designed to ascertain whether honey can improve calcium absorption and/or bone calcium levels. This trial was undertaken at Waikato University between 26 October and 21 December 2005 as was fully funded by Fonterra Brands Ltd.

8.1 INTRODUCTION

As the data from Chapter 7 have shown, there is some limited evidence to suggest that honey may improve calcium uptake and bone calcium levels in calcium-deficient rats compared with sucrose-fed animals. However, as discussed in Section 7.4, the small number of animals used in the previous study as well as the study design employed may have been inadequate to detect any dietary differences. This present study, therefore, does not follow the work of Hamalainen *et al.*, (1990) and Hamalainen (1994) in which a calcium-deficit was artificially induced, but instead uses a different methodology. In the present study, male Sprague Dawley rats were fed a low calcium diet for the duration of the

study, with the diets formulated such that they approximated the composition of a typical New Zealand Diet (New Zealand Ministry of Health, 1999) as this scenario was considered to better represent the typical Western diet. In addition, only two treatment groups (sucrose and honey) were included in this study, each group containing 20 animals in an attempt to increase the power of the study. As with the previous study presented in Chapter 7, lactalbumin rather than casein was used as the protein source to prevent casein phosphopeptides having any effect on calcium absorption (see Section 7.4). In addition, calcium phosphate rather than calcium carbonate was used as the sole calcium source, thereby increasing the amount of calcium that would potentially be unavailable for absorption, as calcium phosphate is relatively insoluble at pH > 7 (Clare and Swaisgood, 2000). Phosphate is also required at relatively high levels in the diet as it is needed with calcium to mineralise bones.

8.2 MATERIALS AND METHODS

8.2.1 Experimental Animals

Forty Sprague Dawley rats, aged approximately 8 weeks (225–250 g) at the start of the trial, were sourced from AgResearch, Hamilton. The trial was carried out at Waikato University as described in Section 2.1.2 with full ethical approval (see Section 2.1.4).

8.2.2 Experimental Diets

Two experimental diets were prepared to contain either 7.9% sucrose (diet # 1) or 10% honey (diet # 2). A rewarewa honey (K*nightia excelsa*) with a high fructose content (45.2%; measured by Agriquality New Zealand Ltd) was used, and the percentage of sucrose used in Diet #1 was based on the finding that the honey contained 21% water (measured using a refractometer).

The powdered diets were prepared by Crop and Food Research, Palmerston North (see Chapter 2). Based upon data from the 1997 National NZ Nutrition Survey, the diets were formulated so that of the total energy content, 15–16% came from protein, 35% came from fat and 45–47% came from carbohydrate. Calcium was incorporated into the diet at a level of 1.6 mg/kg diet. This is lower than the minimal requirement (5 mg/kg) for maximum calcium absorption and bone calcium levels in rodents as reported by The National Research Council (1995) but has been shown to be adequate for growth and bone mineralisation (Bernhart *et al.*, 1969). It was considered that using a less than maximal level of calcium in the diet would allow any differences, if any, in

calcium absorption and bone calcification to be observed between the two dietary treatments. Extra water (21 ml/kg diet) was added to the sucrose diet to account for the water content of the honey. Ten-gram samples were taken from both diets weekly, and the percentage dry matters calculated by drying for 16 hours at 105^{o}C.

In the standard Crop and Food salt mix used in the diets (see Chapter 2), 427 g of $CaHPO_4$ is included per kg of salt mix to give a calcium content of 6.29 mg/kg diet (when added at 50 g salt mix per kg diet). To obtain the low calcium content of the diet used in this study, the salt mix was prepared by adding only 108.62 g $CaHPO_4$ per kilogram of salt mix plus 318.38 g cellulose as filler. The composition of the diets is given in Table 8.1.

Table 8.1: *Composition of Experimental Diets (g/kg)*[a]

Ingredient	Sucrose diet (Diet #1)	Honey diet (Diet #2)
Lactalbumin	160	160
Corn Oil	150	150
Sucrose	79	0
Honey	0	100
Cellulose	50	50
Low Calcium Mineral Mix	50	50
Sugar-free Vitamin Mix	5	5
Starch	445	445
Water	61 ml	40 ml

[a] Supplier information of all ingredients is given in Chapter 2

8.2.3 Experimental Procedures

The animals were weighed upon receipt, and randomly allocated to one of the two dietary treatments. During the experiment, the rats were housed individually in raised stainless steel cages with 6 mm^2 mesh flooring. As required by the Waikato University Ethics Committee, pieces of small grid mesh (~2 mm^2) were affixed with metal twist ties into the back half of each cage so that each rat was able to get off the larger mesh, whilst still allowing urine to pass through. These pieces of smaller mesh were removed and cleaned once a week. Trays containing shavings were slotted into the rack beneath each row of cages. Blotting paper was placed onto each tray (one per cage) to absorb the urine and

collect the spilled diet and faeces. Food jars were loosely affixed into the front of each cage using metal hose clamps. This ensured that feeding occurred near the front of the cage, and that faeces were generally defecated in this area (rats tend to defecate whilst eating). Food, blotters and water were prepared and replaced as described in Section 5.2.3.

On day 56, all rats were anaethetised using CO_2 gas. Dr Ric Broadhurst of AgResearch, Ruakura, Hamilton then removed approximately 5 ml of blood from the heart of each animal via cardiac puncture using 19 gauge needles. These blood samples were allowed to clot, before centrifuging at 3000 *x* g for 10 minutes and removing the serum. The serum samples were sent to the Nutrition Laboratory at Massey University for measurement of serum calcium levels (using the cresol phenolphthalein complexone method, see Chapter 2). All animals were then fully euthanised with CO_2 gas, and the bodies numbered and stored in a freezer.

After approximately two weeks in the freezer, the frozen rat bodies were sent to the Department of Nutrition and Health at Massey University on the overnight courier. There the spine and hind limbs were dissected for DEXA and bone biomechanic analyses (see below).

DEXA

Spines and femurs were stripped of extraneous tissue, leaving about 1 cm of flesh attached. These were submerged in a 1.5 cm deep dish of phosphate buffered saline (PBS). Spines were scanned from L4 to L1, and femurs from the knee to hip joints.

Bone mineral measurements were taken using a Hologic QDR4000 bone densitometer using a pencil beam unit (Bedford, MA, USA). On each day that scans were undertaken, a quality control (QC) scan was taken to ensure that its precision met the required DEXA manufacturer's coefficient of variation. The coefficient of variation (CV) for the QC data was 0.98 – 1.01%. Regional high-resolution scans were performed using a 1.5 mm diameter collimator with 0.31 mm point resolution and 0.5 mm line spacing. Coefficient of variance for the femurs ranged between 0.85% and 0.92% with and without repositioning between scans. These values ranged between 0.98% and 1% for the spine.

Biomechanics

Left femurs were scraped clean of flesh and incubated at 23ºC for 1 hour before the assay, to ensure they were at room temperature for the test. The

length of each femur was measured with an electronic calliper and the midpoint marked with a waterproof pen. Both the vertical and transverse diameter at the midpoint of the femur were similarly determined.

Maximum load, breaking load, maximum deformity (stroke length), breaking stress, breaking strain (the percent of deformation of the femur just prior to the time of breaking), the breaking energy (the amount of energy required to break the femur) and the elastic modulus (force required to bend the bone in the elastic phase of deformation) were determined at the marked midpoint using the Shimadzu Ezi-test (Kyoto, Japan). The femurs were subjected to a three point bending test with the application point of the upper fulcrum positioned midway between the two supporting rods of the testing jig. The supporting rods were spaced 15 mm apart. Load was applied at a constant deformation rate of 50 mm/hour.

Following these analyses the bones were taken to the Nutrition Laboratory at Massey University where they were cleaned and ashed for measurement of bone calcium levels (see Chapter 2 for details).

8.2.4 Statistical Analyses

Two-sample t-tests were carried out on overall and percentage weight gain, total food intake, femur weights, serum and bone calcium levels and all bone-breaking and DEXA measurements. A two-way ANOVA was also performed on the DEXA measurements, comparing the three positions (spine, left femur and right femur) for bone mineral density (BMD) and bone mineral composition (BMC). In addition, a (2 x 9) repeated measures ANCOVA (with Turkeys post-hoc analyses) was performed on the weight gain data using total food intake per animal as a co-variable to determine if there were any diet x time interactions. The analyses were carried out using Minitab and/or Statistica software.

8.3 RESULTS

All animals appeared healthy throughout the 8-week study, and no obvious problems resulting from lowered dietary calcium levels were observed.

No significant differences were observed between the two groups of rats for any of the endpoint measures (see Table 8.2). Figure 8.1 shows the actual mean weight gain over time. As with the other measures, serum calcium levels were comparable for both treatment groups. However, it must be noted that due a problem with several of the centrifuge tubes splitting open during centrifugation,

only 8 serum samples per treatment were able to be retained for analysis. It is therefore possible, but unlikely, that the two treatments may have shown different results had all 40 samples been analysed.

No urinary calcium data was obtained during this trial as metabolism cages were not available for use (metabolism cages have only been used in those studies that were carried out at Crop and Food Research in Palmerston North). An attempt was made to collect the urine in clean, empty trays beneath the mesh-floored cages during the last 24-hour period, however, because of the large surface area of the trays, the small volumes of urine involved and the warmth of the room, evaporation was too rapid and the samples could not be collected.

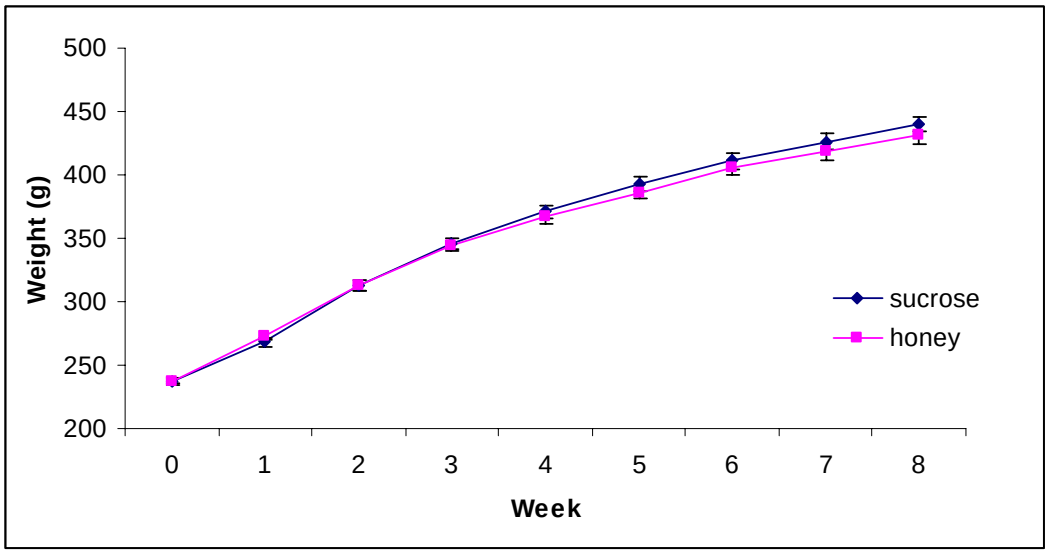

Figure 8.1: *Mean (± SEM; n=20) actual weight gains for rats fed diets containing either 7.9% sucrose or 10% honey over an 8 week period. The sucrose diet had 21 ml of water added per kg of diet such that the moisture content was the same in both diets.*

Table 8.2: *Endpoint measurements (mean ±SD, n= 20 per group) for rats fed the experimental diets (7.9% sucrose or 10% honey (honey is 21% water)) for 8 weeks in a calcium absorption study.*

Result	Unit	Diet	
		Sucrose Diet	Honey Diet
Overall weight gain (actual)	g	202.1 ± 24.4	193.8 ± 22.6
Overall weight gain	%	85.2 ± 11.0	81.8 ± 8.4
Overall food intake	g/8 w	1063.1 ± 82.4	1079.7 ± 99.6
Wet Femur Weight	g	1.10 ± 0.07	1.06 ± 0.08
Dry Femur Weight	g	0.62 ± 0.04	0.61 ± 0.04
Serum calcium levels*	mg/ml	2.74 ± 0.09	2.80 ± 0.08
Bone calcium levels	mg/ml	110.92 ± 5.7	109.78 ± 4.3
Bone Breaking Parameters			
Max Load	N	185.9 ± 19.6	173.4 ± 21.6
Max Stroke	mm	1.82 ± 0.2	1.72 ± 0.15
Break Load	N	185.2 ± 19.3	171.9 ±20.9
Break Stress	mm	75.6 ± 9.0	71.8 ± 12.1
Break Stroke	N/mm^2	1.83 ± 0.16	1.73 ± 0.16
Break Strain	%	17.2 ± 0.2	16.2 ± 2.0
Elastic Modulus	N/mm^2	582.2 ± 104.1	622.7 ± 121.5
Energy	J	0.18 ± 0.02	0.17 ± 0.03
DEXA Parameters			
Bone mineral content			
- spine	g	0.404 ± 0.037	0.405 ± 0.032
- left fumur	g	0.432 ± 0.030	0.418 ± 0.034
- right femur	g	0.434 ± 0.024	0.423 ± 0.027
- left + right femurs combined	g	0.433 ± 0.027	0.420 ± 0.03
Bone mineral density			
- spine	g/cm^2	0.193 ± 0.011	0.193 ± 0.008
- left fumur	g/cm^2	0.211 ± 0015	0.212 ± 0.013
- right femur	g/cm^2	0.212 ± 0.011	0.208 ± 0.010
- left + right femurs combined	g/cm^2	0.211 ± 0.013	0.210 ± 0.012

* Due to a problem with several centrifuge tubes spilling their contents during centrifugation to obtain serum, n=8 for both treatment groups

8.4 DISCUSSION

As the data from this study show, feeding honey did not improve the bioavailability of calcium or increase bone calcium levels when compared with sucrose in rats fed a low calcium diet. These results contrast with the findings of two smaller studies that have shown that honey can improve calcium uptake and retention levels. In 1941 Knott *et al.* demonstrated that infants fed honey-containing formula had calcium retention levels (intake minus urinary and faecal output) that were 1.5–2 times higher than those fed formula containing corn syrup. However, no information was given as to the concentration of honey and corn syrup used, nor whether there was a statistical difference between the two groups. More recently, a study using Sprague Dawley rats showed that absorption of radioactively-labeled calcium was significantly higher two days after a single-dose feeding of 500 or 800 mg honey than in those fed no honey (Ariefdjohan *et al.,* 2005). Importantly, the animals in the study by Ariefdjohan *et al.* (2005) were gavaged with the oral solutions of honey and calcium, and there is no mention of whether the animals were also given access to food during the 2-day period after consumption (this study is published as an abstract only). Eight-week old Sprague Dawley rats consume approximately 22–25 g of food each day (based on the studies presented in this thesis); therefore, the amount of honey fed to the animals in the study by Ariefdjohan (500 and 800 mg) would correlate to approximately 2.2–3.6% of the daily food intake. These levels of honey are substantially less than the 10% used in the present study, but suggest that feeding in one dose may be more effective than *ad libitum* feeding. In human populations, calcium is often consumed in discrete quantities rather than in smaller amounts throughout the day as it is generally limited by the intake of milk and dairy products (milk provides up to 130 mg calcium/100 ml). It is possible, therefore, that feeding honey plus a low level of calcium *ad libitum* in the present study did not allow high enough concentrations of honey to be present in the gut to have had any physiological effect on calcium absorption as the active calcium transport mechanisms would not have been saturated.

Furthermore, having such a low level of calcium ingested at any one time may have meant that it was virtually all absorbed via active transport mechanisms in the duodenum and upper jejunum where the pH would be low enough to keep it soluble. Any dietary factors would therefore have had little or no effect, as these influence solubility of calcium for passive absorption in the lower intestine, and the components of the honey were probably all absorbed before reaching the lower gut. In contrast, feeding the honey and calcium

together in a single meal (as occurred in Ariefdjohan *et al.,* 2005) would have saturated the active transport systems in the duodenum and upper jejunum, with some of the honey and calcium passing into the lower gut allowing a potential increase in passive diffusion of calcium to occur. This may, in turn, have resulted in an increase in calcium absorption overall. As mentioned earlier, no measure of urinary calcium excretion was available as it was deemed to be impossible to collect the urinary calcium because of the warm temperatures in the room leading to evaporation. Since the completion of this study, though, it has been realised that an acid wash of the collection trays followed by volume measurement and calcium analysis would have allowed measurements of excretory calcium levels. Certainly, the lack of this measurement was a flaw in this study, and it should be undertaken in any further studies (in the absence of metabolism cages) to determine what level of absorbed calcium is being retained in the body.

The use of meal feeding (with a large amount of honey and calcium given during one meal) was considered for the present study (and acknowledged as the best trial design); however, it was deemed to not be practical for long-term feeding. With only one person available to feed the 40 animals, it was simply not feasible to take food jars in and out of each cage three times every day for 8 weeks. However, given the findings from the current trial as well as those from the previous authors, it does suggest that any future trial work in this area should look at providing honey and calcium in a bolus rather than as a part of *ad libitum* feeding.

Even if honey could have had an effect on calcium solubility and absorption during *ad libitum* feeding, it is possible that honeys of different floral sources may have differential effects. The honey chosen in the present study was a rewarewa honey with a high fructose content. A study on sugar absorption from mixtures of glucose and fructose has shown that a higher proportion of fructose leads to delayed sugar uptake (MacDonald, 1968), thereby leading to fermentation and acid production in the lower gut that would allow the calcium to remain soluble for longer. However, this may have not occurred. In addition, the level of glucose oxidase activity was measured on this honey and shown to be low. Thus the amount of acid produced directly from the honey would be low (gluconic acid is produced by glucose oxidase) and it unknown whether this gluconic acid production is required for honey to be able to improve calcium uptake. In the work of Ariefdjohan *et al.* (2005), no mention is given as to what the floral source of their honey was. Interestingly, though, these authors did report that a 800 mg glucose/fructose mixture (with proportions of sugar the same

as in the honey they used) also significantly increased calcium absorption compared with animals given no honey, but that this increase in absorption was only half of that reported with an equivalent amount of honey (17.1% vs 33.6%). This finding strongly suggests that non-sugar components in the honey likely contributed to the observed improvement in calcium absorption and bone calcium levels. It is possible that this may have been due to a high level of production of gluconic acid by the honey as increased gastric acidity has been shown to keep calcium soluble for longer (Bo-Linn *et al.*, 1984). In one study that assessed honeys of differing floral sources (Bang *et al.*, 2003) hydrogen peroxide levels were shown to range from 0.3–1.4 mmol/l in 10% honey solutions, to 0.9–2.9 mmol/l in 30% solutions after 30 minutes of dilution (rates decreased again in higher honey concentrations; see Chapter 1.3.2 for a full discussion of factors affecting glucose oxidase activity and rates of hydrogen peroxide in honey). As gluconic acid and hydrogen peroxide are produced by glucose oxidase in a 1:1 ratio (White, 1975a), equivalent concentrations of gluconic should also have been produced. With an 800 mg dose of honey, it would require 2.4–8 ml of fluids in the gut to bring the honey within the range of 10–30%. Ariefdjohan *et al.* (2005) did not give details as to concentration of honey administered (only that it was given as an oral solution) but it is possible that final honey concentrations of 10–30% could have been obtained in the lower gut of the 8-week old animals. Whether such a level of acid production would be sufficient to influence calcium solubility and absorption is unknown; however, as the gut is an anaerobic environment and oxygen is one of the key substrates for glucose oxidase it is unlikely to be an important contributing factor.

It is important to note, though, that glucose oxidase is inactive in honey until the honey is diluted (White *et al.*, 1962) and that enzyme activity continues for only a finite period of time (Bang *et al.,* 2003). The level of gluconic acid that occurs in honey prior to dilution is therefore low, with levels of less than 1% reported by White (1975a). Whether the honey is diluted prior to consuming it could also have a significant impact on its effects on calcium bioavailability. Honey given in an undiluted form as a part of the meal (as in the current study), would probably contain low levels of gluconic acid, and produce only negligible levels upon dilution after ingestion. In contrast, honey given in solution (in concentrations of 50% or less) would likely contain substantially more gluconic acid, the amount present depending on how long the honey had been diluted for prior to being consumed. There is also no mention in the work of Ariefdjohan *et al.* (2005) as to how long the honey solutions were diluted for prior to be fed to

the rats. It is possible, though, that had these solutions been prepared more than 30 minutes prior to feeding, that they may have accumulated sufficient levels of gluconic acid that could have influenced calcium bioavailability in the lower gut, irrespective of the further dilution of the acids that would have occurred *in vivo*.

It is interesting to note also that there was no significant difference in overall percent weight gain in this trial (85% vs 82% for sucrose- and honey-fed rats) as this contrasts with the results from the two earlier weight regulation studies (see Chapters 5 and 6) that showed that honey-fed rats had lower overall weight gains that sucrose-fed rats despite a similar food intake. No direct comparisons can be made between these three studies because of the fact that the animals were different ages (and therefore different starting weights) at the start of each trial; although an indirect comparison is possible. Animals in both the current study and that presented in Chapter 6 had almost identical diet compositions except for the calcium content (the only difference was that the previous study used a honeydew honey with 20% water, whereas the current study used a rewarewa honey with 21% water). A comparison of week 2–6 data from the weight regulation study (where the Week 2 weights were 220–230 g) with the Week 0–4 data of the present study (where starting weights were ~235 g) showed that there were only minimal differences in weight gains between the two studies. In the present study, sucrose-and honey-fed rats exhibited an increase in weight of 56.2% and 54.8%, respectively, during the 4-week period, compared with 61.8% and 60.5% in the weight regulation study. The differences in weight between the two studies are small but may be due to the reduced calcium levels used in the current study. Tordoff and Rabusa (1998) and McCay and Eaton (1947; cited in Tordoff and Rabusa, 1998) have demonstrated that calcium-deprived rats avoid sweet compounds and show a lower intake of sugared water than non-calcium deprived animals. Age-adjusted food intake was reduced by 50–80 g per rat per week in the present calcium trial compared with the weight regulation study suggesting that the animals in the present study may also have exhibited a reduced liking of sweetened food. Potentially, the presence of sufficient calcium and the lack of intake regulation in the weight regulation study may have allowed those animals to consume sufficient extra calories such that any differential effects of sucrose and honey on absorption or metabolism could be observed.

Despite the findings from both the current study and the earlier trial presented in Chapter 7 that honey has no effect on calcium absorption, the limited evidence from other authors suggest that this may still warrant further

investigation. As reported earlier, the results in these two studies do contrast with those that have been obtained in other honey-based studies (Knott *et al.,* 1941; Ariefdjohan *et al.,* 2005). In addition, several studies have demonstrated that a high sucrose diet can alter the calcium balance in humans (Lemann *et al.*, 1970; Thom *et al.*, 1978; Ericssen *et al.*, 1990) and negatively effect levels of both bone (Li *et al.*, 1990; Saffar *et al.*, 1981; Saffar and Markis, 1982; Salem *et al.*, 1992; Tjaderhane and Larmas, 1998) and dentin mineralisation (Tjaderhane, 1996) in rats. The use of honey, therefore, whereas it showed no effect on calcium absorption in short-term feeding, may still be a good alternative to the use of sucrose in food products. Certainly, calcium absorption was no worse with honey than with sucrose in either of the calcium studies investigated in this thesis, and it is possible that longer-term benefits may still be gained. Women, for example, are particularly prone to calcium deficiencies and its related disorders because of the effects that hormonal influences can have on bone calcium loss. As mentioned previously, Goldschmidt and Burkert (1955) have reported that honey acts as if it has hormonal/ neurotransmitter activities. No further research has been undertaken in this area but given the complex composition of honey, it is possible that honey could influence calcium balance at points other than absorption. Studies assessing the effects of honey on both male *and* female subjects are therefore warranted, particularly if real life dietary compositions and regimes can be used. Any data arising from studies assessing calcium bioavailability in rodents needs to carefully interpreted, though, as the intestine of a rodent contains high levels of phytase activity enabling it to hydrolyse phytates in food and absorb calcium down as far as the lower intestine (Gueguen and Pointillart, 2000). More discussion on the relevance of using animal models in this thesis is presented in Chapter 11.

Chapter 9:

The Long-Term Effects of Feeding Honey versus Sucrose and a Sugar-Free Diet on Age-Related Health Parameters

This chapter presents the data from a 12-month feeding study that was undertaken to assess whether long-term feeding of honey might have different effects from those of sucrose and a sugar-free diet. The trial was carried out between February 2006 and February 2007, and was fully funded by Fonterra Brands Ltd. The trial was designed to assess a number of simple age-related parameters (including weight regulation, blood cholesterol levels, blood glucose levels and immunity) as well as a number of cognitive measures. Given the large amount of data generated from this trial, the results have been divided between two chapters. The present chapter will discuss the effects of honey, sucrose and a sugar-free diet on the non-cognitive measures, whilst Chapter 10 will discuss the effects on various aspects of cognition and behaviour.

The main findings of this chapter have been published as follows (see Appendix 3):

Chepulis, L., Starkey, N. (2008) The long-term feeding effects of honey versus sucrose and a sugar-free diet on weight gain, lipid profiles and DEXA measurements in rats. *Journal of Food Science*, 73(1): H1-H7

Chepulis, L. (2007) The effects of honey compared with sucrose and a sugar-free diet on neutrophil phagocytosis and lymphocyte numbers after long-term feeding in rats. *Journal of Complementary and Integrative Medicine.* 4(1), article 8 (Available at: http://www.bepress.com/jcim/vol4/iss1/8)

9.1 INTRODUCTION

Several aspects of health are known to deteriorate as a natural part of the aging process. However, many physiological disorders that can occur in later life, including obesity, cardiovascular disease and Diabetes, are influenced both by genetics and by factors that have accumulated over the span of a lifetime. Most age-related disorders have complex aetiologies and are not due a single factor. In particular, dietary choices often have a significant impact on disease development and progression, and recent evidence suggests that diseases such as obesity, Diabetes, atherosclerosis, hyperlipidemia, insulin resistance and loss of cognitive function can be influenced by long-term dietary changes (see below). Carbohydrate consumption (both the type of carbohydrate and the amount) is recognised as a predominant contributing factor for many of the aforementioned diseases (see below). Thus, the scope of this animal trial was limited to age-related endpoints that could possibly be altered by a change in carbohydrate intake.

Glycemic index (GI), or the postprandial blood glucose level that results after consuming a given quantity of carbohydrate (Jenkins *et al.*, 1981), is the most common way that different carbohydrates can affect disease aetiology. Evidence shows that diets containing substantial amounts of carbohydrate with medium to high glycemic responses (those that break down rapidly during digestion) may actually be detrimental to health because of elevated postprandial hyperglycemia. Specifically, sucrose has been shown to have a direct impact on disease formation and development including atherosclerotic heart disease (Bierman, 1979). Furthermore, the DECODE study, a meta-analysis of more than 20 studies, has shown that increased rates of mortality and morbidity are

associated with high blood glucose levels in both diabetic and non-diabetic patients (The DECODE Study Group, 1999). In addition, links have been made between carbohydrate ingestion and the development of atherosclerosis in non-diabetic subjects (Balkau *et al.*,1998) as well as with colon cancer (Francheschi *et al.*, 2001; Michaud *et al.*, 2005) and breast cancer (Augustin *et al.,* 2001). Epidemiological evidence suggests that different types of carbohydrate may also decrease the risk of cardiovascular disease (Liu *et al.,* 2000; Stampfer *et al.,* 2000) as well as promote satiety, minimise postprandial insulin secretion and increase fat oxidation (Liljeberg *et al.*, 1999; Augustin *et al.*, 2002).

Recent studies have also shown that dietary carbohydrate content can also have a larger impact than previously thought on weight gain (Bell and Sears, 2003), and that sucrose intake can lead to increased weight gain in animal studies (Kanarek and Marks-Kaufman, 1979; Morris *et al.*, 2003; Kawasaki *et al*., 2005). The standard recommendation (besides increasing exercise) for a individual wishing to lose weight is to consume a diet that is low in fat and high in carbohydrate. However, despite the fact that fat intake has decreased in the US during the last 20 years, the number of individuals who are overweight or obese has increased (Bell and Sears, 2003). It has been suggested that this is likely to be due to the carbohydrate component of the diet, as many carbohydrates in the current American diet come primarily from sugars, refined starches and grains (Bell and Sears, 2003). Importantly, short-term feeding studies carried out earlier in this thesis (refer to Chapters 5 and 6) suggest that honey may be able to lead to reduced weight gain compared with sucrose despite a similar carbohydrate content in the diet. Whether this is due to differences in blood glucose response following consumption of the different diets, or due to other, as yet, unidentified substances in honey is unknown at present, but an investigation of its efficacy as a weight reducing carbohydrate during longer-term feeding is warranted. Further support for this investigation is the finding that even when low and high GI diets have differing effects on body weight levels during short-term feeding, this difference often does not continue after consuming the different diets for a longer period of time. Das *et al.* (2007) reported that individuals consuming a calorie restricted diet that was low GI weighed ~ 2kg less than those fed an equivalent high GI load diet, but their body weights were the same as those in the high GI group after 12 months. Thus, regardless of whether honey reduces weight gain compared with sucrose because of differences in glycemic control, antioxidant content or other factors, it is warranted to investigate whether these effects continue to be observed after longer-term feeding.

Cholesterol parameters were also determined in the present study, despite the fact that studies in earlier chapters of this thesis showed the levels to not be different between honey- and sucrose-fed rats. A small number of studies have shown that there is a strong correlation between carbohydrate intake and HDL-cholesterol circulatory levels (Frost *et al.*, 1999; Luscombe *et al.*, 1999; Buyken *et al.*, 2001; Ford and Liu, 2001; Liu *et al.*, 2001),and that sucrose can induce postprandial hypertriglyceridemia (Grant *et al*., 1994) elevated triacylglycerol levels (Fried and Rao, 2003) and elevated total cholesterol and triglyceride levels (Saku et al., 1996). Again, there appears to be little information available as to how a mixture of high and low GI sugars (like glucose and fructose in honey) might impact on physiological measures such as cholesterol. For this reason, and given the ease with which the samples can be measured, these too were deemed to be key measures in this study.

In addition, diets that contain large quantities of carbohydrates can result in prolonged hyperglycemia, and one of the ways that this can have detrimental effects is through the induction of oxidative stress, with chronic or excessive increases in oxidant production adversely affecting cellular physiology and function (Mezetti *et al.,* 2000; Chang *et al.,* 2003; King and Locken, 2004). In particular, there is a lot of evidence to suggest that oxidative stress is involved in the aetiology of several diabetic complications (reviewed by Chang *et al.,* 2003). A failure in insulin-stimulated glucose uptake by fat and muscle results in blood glucose concentrations remaining high. As a result, glucose uptake by insulin-*independent* tissues increases, and oxidant production increases due to an upregulation of several pathways, including protein kinase C and mitochondrial NADPH oxidase activation (Moskovitz *et al.*, 2002). This, in turn, leads to increased lipid peroxidation, amide-type adduct formation, oxidised tyrosine moieties and advanced glycation end-products (AGEs) (Osawa and Kato, 2005). Importantly, accumulating evidence suggests that oxidative damage plays a major role in the development and progression of other age-related disorders including cancers, mitochondrial dysfunction, chronic inflammation, inschemia, degenerative arterial and autoimmune diseases (Davies *et al.*, 2001). The critical targets that are affected by oxidative damage during aging have not been specifically identified, but DNA damage has been shown to be involved (Richter *et al.*, 1988) as has protein carbonyl formation (Moskovitz *et al.*, 2002). Oxidative damage is also known to play a significant role in cognitive decline in older individuals: this is discussed, in length, in Chapter 10.

Immune function is also known to decline in older age (Makinodan and Kay, 1980), and limited research suggests that this is due to dysregulation of the immune system rather than a decrease in immune response (Lesourd, 1997). Aging in humans is associated with a decrease in cell-mediated immunity, particularly a reduction in peripheral blood lymphocyte counts, and with changes in T lymphocyte subpopulations (Lesourd, 1997; Castle, 2000). Similarly, neutrophil activity has also been shown to decline in older populations (Wenisch *et al.*, 2000; Butcher *et al.*, 2001). The latter may be of particular importance as neutrophils are considered to be the "first line of defense", these being the leukocytes that respond most rapidly to a pathogenic invasion.

Recently, it has been reported that the decline seen in immune function in older age may be related to increased levels of oxidative stress, with antioxidant intake being able to improve immune function (De La Fuente *et al.*, 2005). Thus it is suggested that honey may be able to improve age-related immune function due to its antioxidant content, and due to it possible reducing hyperglycemia-induced oxidative damage. In addition, research has shown that honey can have a prebiotic effect (see Chapter 1) and that prebiotics do have the ability to enhance immune responses (Yamada *et al.*, 1999; Schley and Field, 2002). Limited research has demonstrated that honey can have an effect on immune function. In *in vtiro* studies it has been shown to stimulate the release of tumour necrosis factor-α in unprimed monocyte cells (Tonks *et al.*, 2001, 2003), and increase proliferation of B- and T-lymphocytes and neutrophils *in vitro* (Abuharfeil *et al.*, 1999). In addition, honey has been demonstrated to stimulate antibody production during primary and secondary immune responses against thymus-dependent and thymus-independent antigens (Al-Waili and Haq, 2004). Thus, the immune effects resulting from feeding sucrose, honey and a sugar-free diet were also compared in this study.

The aim of this study was to assess whether feeding honey, sucrose or a sugar-free diet long-term would have any effects on age-related parameters. Weight gain, food intake, cholesterol and blood glucose levels were determined, as well as some simple immunity measures (neutrophil phagocytosis and lymphocyte counts) and a measure of overall oxidative damage (AGEs) in aortic collagen. Percentage body fat levels were determined using DEXA analyses to ascertain whether any differences in weight gain were attributable to changes in lean or fat body mass. Furthermore, because a number of parameters can easily be assessed during a single DEXA scan without any increase in cost (and because these endpoints gave inconclusive findings in studies presented in

previous chapters) , bone mineral density and bone mineral composition levels were also measured during this study.

9.2 MATERIALS AND METHODS

9.2.1 Experimental Animals and Housing

Fifty five rats, aged approximately 8 weeks at the start of the trial, were obtained from AgResearch, Hamilton. The trial was carried out at the University of Waikato as described in Section 2.1.2 with full ethical approval (see Section 2.1.4).

9.2.2 Experimental Diets

A honeydew honey (HD19) with a high antioxidant content (TEAC = 3.1 mmol/l) was chosen for use in this study (see Chapter 3 for details on the determination of TEAC levels). Three experimental diets were prepared to contain no sugar (Diet # 1), 7.9% sucrose (Diet # 2) or 10% honey (Diet # 3). All diets were prepared to contain a minimum of 5% water. An additional 21 ml of water was also added to each kilogram of the non-honey diets to account for the fact that the honey contained 21% water (measured using a refractometer).

In this study, the diets were prepared such that they approximated the composition of a typical New Zealand diet. Based upon data from the 1997 National Nutrition Survey (New Zealand Ministry of Health, 1999), the diets were formulated so that of 100% total energy, 15–16% came from protein, 35% came from fat and 45–47% came from carbohydrate. To satisfy the requirements from Fonterra that milk be incorporated into the diets, skim milk powder was added to the diets at a level of 8% of the total daily kilojoule intake based on a dietary kilojoule content of 18 kJ/g (this value being obtained from the diets prepared in Chapters 6 and 8 as they were also formulated to contain the same level of energy from protein, fat and carbohydrate). This level of skim milk powder (8%) was chosen as it is equivalent to approximately 350–400 ml of milk or dairy products in an average person's daily diet (assuming a total energy intake of 11000-12000 kJ/day). This results in the rodents consuming approximately 1.9–2.3 g skim milk powder per day (assuming an intake of 20–25 g food per day). The skim milk powder used in these diets was sourced from Fonterra Research, Palmerston North and had the following specifications: energy 1520 kJ/100 g, protein 36.1 g/100 g and fat 0.8 g/100 g. These values were used to determine

the quantities of additional fat, protein and carbohydrate required in the three diets as follows:

Protein in diet = 34.3 g (from skim milk powder) +120 g (from casein) = 154.3 g
= 154.3 g x 17 kJ/g
= 2 623 kJ
= approximately 15.4% of the total energy in the diet

Fat in Diet = 0.8 g (from skim milk powder) + 160 g = 160.8
= 154.8 g x 37 kJ/g
= 5 949 kJ
= approximately 35% of the total energy in the diet

Carbohydrate in Diet = 51.4 g (lactose in skim milk powder) + 79 g (sugar/honey/amylose) + 365 g(starch)
= 495.4g
= 495.4g x 17 kJ/g
= 8 429 kJ
= approximately 48% of the total energy in the diet

To enhance the possible levels of oxidative damage that may occur in these animals, the diets were prepared using pre-used cooking oil rather than virgin oil as the source of fat. The cooking oil was sourced from various commercial kitchens in Palmerston North. As it was not possible to obtain sufficient pre-used oil for the full 12 month period, the oil was obtained in batches (enough for approximately 3 months of diet) and well mixed prior to inclusion in the diets. This ensured that whereas there may be slight differences over time, these would be standard across all three diets, and there would be no differences in the composition of the oil between diets at any one time.

A low GI starch product (amylose) was used in the sugar-free diet as a replacement for the sucrose/honey rather than standard starch that has a high GI. This allowed any effects in the honey-fed rats to therefore be attributed to the lower GI component of the diet (if the response was also similar in rats fed a sugar-free diet) or to other components of the honey (if a different response was observed in rats fed a sugar-free diet).

Salt and vitamin mixes were prepared as described in Chapter 2; however, the mineral mix was modified to reflect the amounts of calcium (1.33 g/100 g) and phosphate (1.05 g/100 g) added to the diet from the skim milk powder (cellulose was added instead to make up the final weight). All other mineral levels in the skim milk powder were minimal and therefore not altered in the prepared mineral mix. The composition of the diets is given in Table 9.1.

Table 9.1: *Composition of Experimental Diets (g/kg)*[a]

Ingredient	Sugar-Free Diet	Sucrose diet	Honey diet
Skim milk powder	95	95	95
Casein	120	120	120
Used oil	160	160	160
Amylose	79	0	0
Sucrose	0	79	0
Honey	0	0	100
Cellulose	50	50	50
Modified mineral Mix	50	50	50
Sugar-free Vitamin Mix	5	5	5
Starch	365	365	365
Water	76 ml	76 ml	55 ml

[a] Supplier information of all ingredients is given in Chapter 2

Sub-samples of the three diets were sent to the Nutrition Laboratory at Massey University for measurement of their energy content by bomb calorimetry (see Chapter 2). The percentage dry matter of these diets was determined prior to commencing the study, and once a month thereafter for the remainder of the trial. The percentage dry matter in the diets was calculated by drying weighed samples in triplicate at 105^{o}C for 16 hours. Percentage dry weights were used to calculate actual food intake and to ensure that there were no differences in moisture content during storage. Fresh diets were prepared by Crop and Food Research every month and stored at -16^{o}C until required. All bags of diet were allowed to warm to room temperature for 3 hours before feeding to the animals.

9.2.3 Experimental Procedures

Animals were weighed upon receipt, and the five smallest and five largest housed together in two large cages. These "spare" animals were fed on standard rat pellets and kept as practice animals for the various cognitive tasks (see Chapter 10). The remaining animals were randomly allocated to one of three experimental diets and housed individually in standard rat cages that contained shavings. The cages were all kept in one room of the Animal Behaviour building, with three levels of cages on shelves around three walls of the room. A 10–12 cm long piece of 90 mm PVC tubing was placed into each cage to allow the animals a place to "hide". Enrichment devices (including small plastic containers, pegs, sticks and pieces of doweling) were routinely added to the cages to give the animals something to play with to prevent boredom. Food and water were prepared and replaced as described previously.

9.2.3.1 Weight Gain and Food Intake

Animal weights were recorded every 1–2 weeks throughout the 12 month period and food intake was assessed every two months (including the start of the study period). For assessment of food intake (refer to Section 8.2.3 for details) the animals were removed from their home cages and placed in raised stainless steel cages in another room of the same building. Animals were allowed to acclimatise to the new cages for 4 days before beginning the food intake assessment. During the acclimatisation period, all animals had *ad libitum* access to their food and water but intake was not recorded. Food, blotters and water were then prepared and replaced as described in Section 5.2.3. On completion of the food intake assessment, each rat was removed from the raised cage and placed back into a clean home cage.

9.2.3.2 Sample Analyses

On Days 364 and 365 (half of each treatment group on each day), the rats were anaethetised using CO_2 gas. Dr Ric Broadhurst of AgResearch, Hamilton then performed a cervical dislocation on each animal and removed approximately 10 ml of blood from the heart of each animal via cardiac puncture using 19 gauge needles. Approximately 2 ml of the removed blood was added to an EDTA blood collection tube (for measurement of glycated haemoglobin (HbA1c)), 2 ml to a heparin blood collection tube (for measurement of neutrophil phagocytosis – see below) and the remainder added to a standard Vacutainer. These latter blood samples were allowed to clot, then centrifuged at 3 000 x *g* for 10 minutes and

the serum removed (for fasting lipid profiles). Both the EDTA-collected blood and the serum were then taken to the laboratory at Waikato Hospital for their respective analyses (refer to Chapter 2 for details). Samples of abdominal aorta (1–1.5 cm) were removed, cleaned in PBS buffer and stored at -70^{o}C. The animal bodies were stored in a -20^{o}C freezer for further analysis.

DEXA

After approximately 3 weeks in the freezer, the rat bodies were sent to the Department of Nutrition and Health at Massey University by overnight courier where they were analysed for percentage body fat and bone mineralisation levels using DEXA. Bone mineral measurements were taken using a fan beam Hologic QDR Discovery bone densitometer (Bedford, MA, USA). On the day preceding each set of scans, a quality control scan of an anthropomorphic spine phantom was taken to ensure the unit's precision. The quality control values fell within 1.5% of the mean. A small step phantom was also scanned as quality control for body composition measurements. The whole rat bodies were scanned using collimator size 10.24 X 0.10 with point resolution of 0.064 cm and 0.1512 cm line spacing. Regional high-resolution scans of the lumbar spine, and the right and left femurs were performed using 5.69 X 0.03 collimator. Point resolution and line spacing were 0.0311 cm. Rats were positioned supine with right angles between the spine and femur, and between femur and tibia.

For the *ex vivo* scans the left femurs were stripped of extraneous tissue, leaving about 1 cm of flesh attached. These were submerged in a 1.5 cm deep dish of PBS. Regional high-resolution scans of the left femurs were performed using 5.69 X 0.03 collimator. Point resolution and line spacing were 0.0311 cm. The bone mineral density coefficients of variation for the femurs ranged between 1.87% and 0.45% with and without repositioning between scans respectively. These values ranged between 1.91% and 0.85% for the spine, and 0.74% and 0.55% for the whole body.

Assay of Neutrophil Phagocytosis and Lymphocyte Numbers

Heparin-collected blood was taken to AgResearch, Hamilton and assayed for neutrophil phagocytosis activity (within 2 hours of collection) using a method supplied by Fran Wolver at Massey University. Briefly, 100 μl of whole blood was added to a pre-cooled FACS tube in an ice bath. A sample (50 μl) of fluorescein isothiocyanate (FITC)-labelled *E.coli* (prepared in-house using non-oponised K12 strain *E.coli* and Fluorescein conjugate W-2861) containing 4 x 10^6 bacteria was

then added to each tube (except the control) and the tubes incubated at 37°C for 10 minutes (the control tube was left in the icebath). After incubation, 50 μl of FITC-labelled *E.coli* was added to the control tube. All tubes were then lysed with FACS lyse buffer (BD Biosciences Catalog number 349202) and the tubes incubated at room temperature in the dark for 10 minutes. The tubes were then centrifuged at 1200 rpm for 10 minutes at 18°C, the supernatant discarded and the pellets re-suspended in 1ml each of PBS buffer. The tubes were again centrifuged as described and the supernatant discarded. Each sample was then re-suspended in 500 μl running buffer (1% paraformaldehyde in PBS), and 50 μl Trypan blue (0.4% in PBS) added to each tube. These samples were then run on a Becton Dickenson Flow Cytometer and the percent neutrophil phagocytosis and percent leukocytes that were lymphocytes determined using standard in-house methodologies.

Measurement of Advanced Glycation End-products (AGE)

The assay used was based upon the protocol of Makita *et al.* (1992). In summary, aortic samples (collected at the time of euthanisation) were slowly thawed from -70°C during a 48 hour period (24 hours at -20°C, followed by 24 hours at 4°C). Once thawed, the tissues were scraped with a scalpel to remove adherent fat, and then rinsed in PBS buffer (pH 7.4). The samples were blotted dry and finely minced with scissors, with the wet weights recorded. Lipids were extracted from the aortic samples with a 1:1 mixture of acetone/chloroform by shaking overnight at 4°C (50 volumes (w/v) of extractant was used as detailed in Dunn *et al.*, 1991). Samples were then freeze-dried for 24 hours, weighed and resuspended in 0.2 mol/l $NaPO_4$ buffer (pH 7.4) to a final concentration of 10 mg/ml. Collagenase (CO130, Sigma) was added to each sample tube at a ratio of 1:100 (w/w) and the mixture incubated for 48 hours at 37°C with mild shaking. One drop of toluene was added to each tube to maintain sterility during incubation. The digested samples were then centrifuged at 15 000 *x g* and the clear supernatants removed.

The collagen AGE products were determining by measuring fluorescence, (Flurostar Optima, BMG Laboratories) with excitation:emission at 390:460 nm (malondialdehyde (MDE) protein adducts) and 355:460 nm (4-hydroxy-2-nonenal (HNE) protein adducts). The test samples were measured at a protein concentration of 10 mg/ml, with the samples all tested in duplicate, or in triplicate where sample volume allowed. A sample blank (buffer only) was also run, and

these values subtracted from all test values. The values were expressed as relative fluorescent units.

9.2.4 Statistical Analyses

A one-way ANOVA was performed on all parameters to determine if there were statistical differences between the three dietary treatments. In addition, a (3 x 37) repeated measures ANCOVA was performed on the weight gain data using total food intake per animal as a co-variable to determine if there were any diet x time interactions. Post-hoc analyses included Turkeys analyses, and all analyses were carried out using SPSS software (version 12).

9.3 RESULTS

Unfortunately, despite assurances from staff at Agresearch that all the rats supplied were male, two of the rats were actually female as they gave birth within two weeks of commencing the trial. These two animals were removed from the study, and the babies grown and kept for other laboratory work at the University of Waikato. For this reason the initial sample size for the remainder of the trial was 14, 14 and 15, respectively for sugar-free-, sucrose- and honey-fed rats.

All animals remained healthy throughout the 12-month period and no obvious signs of illness were observed. In the first few months as the rats got bigger, a few animals (5–6) routinely tried, and succeeded, at escaping from their cages, though they were never out of their home cage for more than 12 hours. More enrichment devices were placed into their cages to try and alleviate the boredom, and the cage lids were weighted down with concrete tiles or pieces of wood. However, in week 44, one animal (rat #3, sugar-free group) managed to escape from its cage on a top shelf and was found dead on the floor the following morning. No obvious cause of death was determined, and its death was deemed to likely to have been due to the fall. A second animal (rat #43, honey group) was found dead in its cage in week 46. Dissection revealed that this animal had a large abdominal tumour, No further investigations were carried out; although tumour formation is common in Sprague Dawley rats so it was considered to most likely not be diet-related.

9.3.1 Weight Gain and Food Intake

The results of this study (presented in Table 9.2) suggest that honey and sucrose do have different effects on weight gain in adult rats. The mean overall

percentage weight gain in honey-fed rats was similar to that observed in rats fed a sugar-free diet, and was significantly reduced compared with those fed sucrose after 52 weeks ($p < 0.05$). Overall percentage weight gain was also significantly reduced in rats fed the sugar-free diet compared with the sucrose-fed rats ($p < 0.01$). A repeated measures ANCOVA analysis of weight revealed a significant interaction of diet * time ($p < 0.001$), and a significant effect of diet. Weight measurements were significantly different between sugar-free and sucrose-fed rats ($p < 0.001$) and sucrose- and honey-fed rats ($p < 0.05$). Figure 9.1 shows the overall percentage weight gain during the duration of the study, and Figure 9.2 shows the mean percentage weekly weight gains for all three treatments.

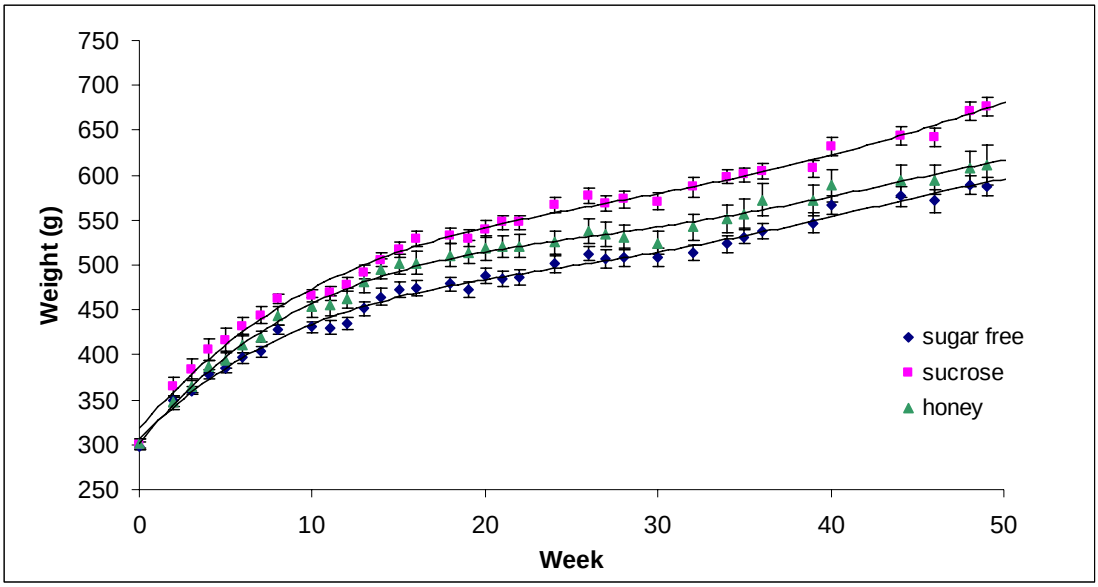

Figure 9.1: *Actual mean weight values (± SEM) for rats fed diets that were either sugar-free, or contained 7.9% sucrose or 10% honey. All diets were formulated to contain equivalent amounts of honey/sugar/amylose and water (the honey was 21% water).*

Food intake was assessed during seven week-long periods, each measurement period being two months apart. No significant differences were observed between treatments at any of these endpoints, nor in the weekly or overall 7-week kilojoule intake (see Table 9.2).

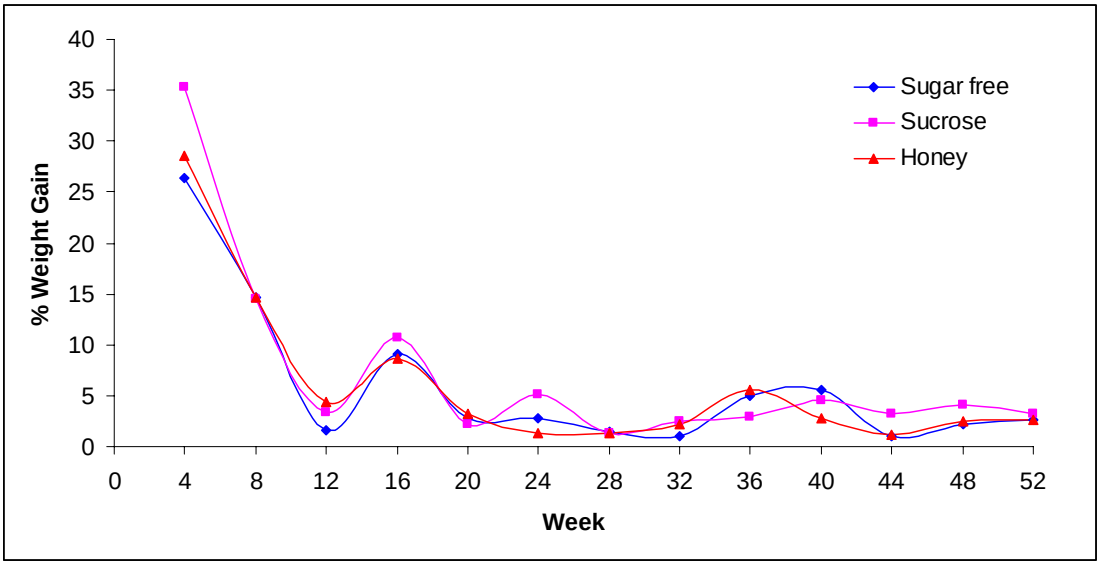

Figure 9.2: *Mean percentage 4-weekly weight gain (based on the previous months weight) for rats fed diets that were either sugar-free, or contained 7.9% sucrose or 10% honey. All diets were formulated to contain equivalent amounts of honey/sugar/amylose and water (the honey was 21% water).*

9.3.2 Blood Glucose (HbA1c) and Cholesterol Levels

HbA1c levels were significantly lower in rats fed the honey diet compared with those fed the sucrose diet ($p < 0.001$), but there were no significant differences observed between the sucrose and sugar-free treatment groups (see Table 9.2). No significant differences in levels of total-cholesterol, LDL-cholesterol or triglycerides were observed after 52 weeks of feeding between any of the respective treatments; however, HDL-cholesterol was shown to be significantly higher (16–21%) in honey-fed rats than in those fed the sucrose ($p < 0.05$) or sugar-free ($p < 0.01$) diets (see Table 9.2).

Table 9.2: *Mean data (mean ± SD) for rats fed diets that were either sugar-free, or contained 7.9% sucrose or 10% honey after 12 months of feeding. HbA1c = glycated haemoglobin; HDL = high density lipoprotein; LDL = low density lipoprotein*

Result	Unit	Diet Sugar-free (Diet # 1), n=14	Sucrose (Diet # 2), n=14	Honey (Diet # 3), n=15
Overall weight gain	%	102.5 ± 19.7 †	130.6 ± 26.7*	107.2 ± 13.8 †
Overall food intake[a,b]	g/7w[f]	1246.4 ± 85	1243.6 ± 111	1244.8 ± 89
Overall kilojoule intake[c]	kJ/7w[f]	23182 ± 1580	23019 ± 2053	22730 ± 1620
Faecal Output [b]	g/7w[f]	131.4 ± 6.9	124.7 ± 12.6	133.1 ± 10.9
HbA1c	mmol/l	4.07 ± 0.17	4.19 ± 0.14	3.97 ± 0.12†
Total Cholesterol	mmol/l	2.79 ± 0.45	2.98 ± 0.59	3.12 ± 0.37
LDL Cholesterol	mmol/l	0.09 ± 0.09	0.10 ± 0.14	0.06 ± 0.14
HDL Cholesterol	mmol/l	2.32 ± 0.33	2.44 ± 0.51	2.82 ± 0.30*†
Triglycerides	mmol/l	0.85 ± 0.24	0.96 ± 0.34	0.86 ± 0.33
Neutrophil phagocytosis[d]	%	51.7 ± 11.7†‡	79.2 ± 11.5*	74.7 ± 14.6*
% lymphocytes [d,e]	%	29.5 ± 8.0†‡	40.1 ± 10.8*‡	53.0 ± 6.6*†

* significant *vs* sugar-free diet; † significant *vs* sucrose diet; ‡significant vs honey diet ($p < 0.05$)
[a] Food intake corrected for percentage dry matter (determined by heating duplicate samples for 16 hours at 105°C)
[b] Data collected from the 7 weeks of food intake assessment only
[c] kilojoule content of the diets were 18.6, 18.5 and 18.3 kJ/g for sugar-free, sucrose and honey diets, respectively.
[d] Due the cost of the assays, the sample size of each group was reduced to 12 for this test.
[e] Percentage of leukocytes that were lymphocytes. The data was generated by the flow cytometer; actual counts were not generated.
[f] 7w data is actually the total of seven separate one-week periods obtained at 2-monthly intervals.

9.3.3 DEXA Scans

The full body scans by DEXA revealed a small number of differences in the body composition of the animals in the three dietary treatments (see Table 9.3). Lumbar spine area (total bone surface area) was significantly greater in both sucrose- and honey-fed rats compared with those given a sugar-free diet (both $p < 0.05$); and whole body bone surface area ($p < 0.05$) and bone mineral composition ($p < 0.01$) were significantly higher in rats fed sucrose compared with those given the sugar-free diet.

Table 9.3: *Mean DEXA Data (mean ± SD) for rats at the end of feeding for 12 months on diets that were either sugar-free, or contained 7.9% sucrose or 10% honey.*

Result	Unit	Diet		
		Sugar-Free (Diet #1)	Sucrose (Diet #2)	Honey (Diet 3)
Left Femur				
Area	cm^2	1.94 ± 0.07	2.04 ± 0.11	2.07 ± 0.12*****
BMC	g	0.59 ± 0.04	0.64 ± 0.07	0.65 ± 0.07
BMD	g/ cm^2	0.30 ± 0.01	0.31 ± 0.02	0.31 ± 0.02
Right Femur				
Area	cm^2	1.98 ± 0.13	2.02 ± 0.11	2.09 ± 0.07
BMC	g	0.60 ± 0.05	0.64 ± 0.07	0.65 ± 0.05
BMD	g/ cm^2	0.30 ± 0.01	0.32 ± 0.02	0.31 ± 0.01
Lumbar Spine				
Area	cm^2	2.67± 0.07	2.84 ± 0.14*****	2.83 ± 0.15*****
BMC	g	0.65± 0.04	0.71 ± 0.11	0.72 ± 0.07
BMD	g/ cm^2	0.24 0.01	0.25 ± 0.03	0.25 ± 0.02
Whole Body				
Area	cm^2	82.9 ± 5.3	90.1 ± 6.9*****	85.1 ± 3.9
BMC	g	12.5 ± 0.6	14.4 ± 1.6*****	13.7 ± 0.65
BMD	g/ cm^2	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01*****
Percent Fat	%	26.5 ± 5.4	34.7 ± 9.1‡	25.5 ± 6.4†

***** significant *vs* sugar-free diet; † significant *vs* sucrose diet; ‡significant vs honey diet ($p < 0.05$)
BMC = bone mineral composition, BMD = bone mineral density.

Honey-fed rats exhibited a slight, but statistically significant, increase in mean whole body bone mineral density (BMD) (0.161 g/cm^2) compared with those fed a sugar-free diet (0.151 g/cm^2; $p < 0.01$). Sucrose fed rats had a BMD of 0.159 g/cm^2; however, this was not significantly different from rats fed either honey or a sugar-free diet.

Mean total percent body fat was significantly higher in sucrose-fed rats (34.7%) than in honey-fed rats after 12 months (25.5%; $p < 0.05$). No significant differences were observed between sucrose-fed rats and those given a sugar-free diet, although percentage body fat levels were similar for animals given honey and the sugar-free diet (26.5%).

9.3.4 Neutrophil Phagocytosis and Lymphocyte Populations

The percentage of neutrophils that exhibited fluorescence resulting from phagocytosis was similar for sucrose- and honey-fed rats (79.2% and 74.7%) and both of these treatments were significantly higher than rats fed the sugar-free diet (51.7%; both $p < 0.0001$). The percentage of leukocytes that were lymphocytes also differed significantly between all three diets (see Table 9.2). The lymphocyte percentage was nearly twice as high in honey-fed rats compared with those fed the sugar-free diet ($p < 0.0001$), and nearly 13% higher than those fed sucrose ($p < 0.01$). The percentage lymphocyte values for rats fed the sucrose and sugar-free diets was also significantly different at $p < 0.0001$ (Table 9.3).

9.3.5 Measurement of Advanced Glycation End-products

Mean levels of MDA oxidative damage in the aortic collagen were significantly higher ($p < 0.05$) in sucrose-fed rats (55390 arbitrary fluorescent units) compared with either honey-fed rats (50661 units) or those fed a sugar-free diet (50974 units) after 12 months. HNE oxidative damage was not detectable in any of the three dietary treatments investigated.

9.4 DISCUSSION

As the results from this study demonstrate, there do appear to be health benefits associated with replacing sucrose in the diet with honey for a prolonged period of time. As with the shorter-term feeding studies presented earlier in this thesis, weight gain was substantially reduced in honey-fed rats compared with those given a sucrose-based diet. Honey-fed rats had mean overall weight gains that were similar to those of animals fed the sugar-free diet, and this agrees with the findings in Chapter 6, despite small differences in diet composition between

the two trials. Importantly, these comparable data suggest that the weight-regulating property of honey is not restricted to only young animals (animals were aged 6–12 weeks in the earlier trial) but instead may occur throughout the lifetime of the animal. In Chapter 6; however, reduced weight gain occurred in honey-fed rats even though the overall 6-week food intake was significantly higher in these animals compared with those fed the sugar-free diet. In contrast, food intake in the current study appears to be similar for all treatments groups throughout the duration of the study. Food intake in the current study was assessed during seven week-long periods and not for the total duration of the study, as occurred in the earlier study. Due to the need to house the animals individually long-term, it was not ethically viable to use food assessment cages for everyday housing. These cages are smaller than those required by the Waikato University Ethics Committee, and as they have a mesh floor, they are unsuitable for long-term use. However, given the standard housing conditions used throughout the 12 month period, it is unlikely that food intake would have varied between the three dietary treatments during the times that food intake was not measured. In Chapter 6, food intake (and total kilojoule intake) was significantly higher in the sucrose, mixed sugars and honey groups compared with the sugar-free animals. Thus, it was suggested that honey-fed rats had a lower weight gain per kilojoule consumed (compared with animals fed either the sugar-free or sucrose diets), these animals somehow diverting the extra consumed kilojoules away from fat storage. In the present study, however, kilojoule intake was the same for all three dietary treatments. This suggests that both the sugar-free and honey-based diets demonstrated a reduced weight gain/kJ compared with the sucrose-fed group.

It must be noted, though, that the sugar-free diet prepared in the current study was not entirely sugar-free as it contained nearly 10% skim milk powder. Skim milk powder contains, on average, 52% lactose; therefore, even the sugar-free diet contained approximately 5% sugars by weight. This may have been sufficient to increase the sweetness of the diet, thereby improving palatability. An increase in palatability may account for why food intake was not reduced in rats fed the sugar-free diet in the current study, whereas it was in the trial presented in Chapter 6. However, similar kilojoule intakes should have potentially led to similar levels of weight gain and clearly this was reduced in animals fed the sugar-free and honey diets compared with those fed sucrose. This suggests that kilojoule intake is not solely responsible for the weight gains (or lack thereof) observed in this study.

Importantly, the three diets only differed in the content of the amylose, sucrose, and honey. Without having accurate measures of the GI of the different diets it is impossible to speculate as to whether differences in GI may have been responsible for the differences seen in weight gain, but it is unlikely given that the majority of the carbohydrate content (starch plus lactose) was the same across all treatments). However, that said, the data does suggest that there may be an differential effect of honey and sucrose on blood glucose as plasma HbA1c levels were reduced in both sugar-free and honey-fed rats compared with those fed the sucrose diet (although this difference did not reach significance for the animals fed the sugar-free diet). Glycated haemoglobin (HbA1c) is routinely used as a measure of long-term serum glucose regulation as it is easily quantifiable, with the haemoglobin occurring in large quantities in the blood. However, glycation is a non-enzymic, free-radical process, and the presence of antioxidants can reduce the frequency of this reaction, thereby reducing the measurement of HbA1c (Osawa and Kato, 2005). The honey used in this study was specifically chosen for its high antioxidant content (see Chapter 10 for more details) and it is possible that the antioxidant content of the honey, rather than any differential glycemic effects was responsible for the reduced HbA1c level detected in these animals. In addition, it may explain why the honey-fed rats had a lower mean HbA1c levels than those fed the sugar-free diet (although that these two groups were not significantly different). HbA1c levels were not reduced in honey-fed rats (nor in any of the other treatment groups) in the trial in Chapter 6, and it is difficult to explain why this is so, given that similar honeydew honeys with similar antioxidant contents (TEAC = 2.7 and 3.1) were used. It could be that the earlier trial simply did not run for a long enough period of time to be able to detect a difference (HbA1c is a marker of the level of haemoglobin glycation occurring over the previous 4–12 weeks), or that other factors (either diet- or age-related) affected the level of glycation that occurred.

Interestingly, cholesterol parameters were also altered in this study, with honey-fed rats exhibiting HDL-cholesterol levels that were 15–20% higher than those fed the sugar-free and sucrose diets. No other long-term feeding studies have investigated the effects of honey on lipid profiles; however, short-term animal feeding studies have shown no increases in HDL-cholesterol levels, either compared with baseline levels or with other dietary treatments (see Chapter 6 and Al-Waili, 2004b). It is possible that the changes in HDL-cholesterol observed in the present study occurred gradually over a prolonged period of time, rather than occurring in only a few weeks. As mentioned earlier, there is a strong link

between high GI diets and low HDL-cholesterol levels (Frost *et al.*, 1999; Luscombe *et al.*, 1999; Buyken *et al.*, 2001; Ford and Liu, 2001; Liu *et al.*, 2001); thus, we can speculate that the honey-diet may have contributed to the increase in HDL-cholesterol levels because of differences in glycemic response. However, as HDL-cholesterol levels were comparable between the low GI-sugar-free diet and the high GI-sucrose diet, it is unlikely that dietary GI played a significant role. Several factors have been reported to improve HDL-cholesterol levels in humans, including aerobic exercise, weight loss, cessation of smoking and supplements such as omega 3 fat, monounsaturated fat and nicotinic acid (Drexel, 2006) although it is hard to see how any of these factors may be relevant to the current study, except maybe the weight loss. Van Gaal *et al.* (1997) reported that a weight reduction of as little as 5–10% can significantly improve HDL-cholesterol levels in overweight individuals. Similarly, several authors have reported specific improvements in HDL-cholesterol levels in both men and women after 12–18 months of weight-reducing diets (Wood *et al.*, 1991; Williams *et al.*, 1994; Wing and Jeffery, 1995; Stefanick *et al.*, 1998). It is possible, therefore, that the increase in HDL-cholesterol levels seen in honey-fed rats was directly related to the reduced levels of body weight compared with animals fed the sucrose diet. However, if the improvement in HDL-cholesterol was purely due to reduced body fat levels, then animals fed the sugar-free diet should also have demonstrated increases in HDL-cholesterol levels compared with sucrose-fed animals, and this did not occur. Thus, it would seem that there are other factors responsible for the improvement in HDL-cholesterol levels observed in honey-fed rats.

It is important to note, though, that the implication of increased HDL-cholesterol levels in honey-fed rats is still significant, regardless of the means by which it occurred. In clinical trials there have been strong associations seen between low HDL-cholesterol levels and the increased risk of cardiovascular disease (Drexel, 2006). Several large studies (Carlson and Rosenhammer, 1988; Rubins *et al.*, 1999; Canner *et al.*, 2003) have also investigated the use of HDL-cholesterol-raising agents as a therapeutic strategy for improving cardiovascular outcomes in high-risk populations and the findings have been impressive. The risk of death, myocardial infarction, stroke or revascularisation was reduced by up to 90% with only minimal improvements in HDL-cholesterol levels. The ability to improve HDL-cholesterol through simple dietary means is therefore a valuable tool, particularly in individuals that are high-risk for the development of cardiovascular disease.

The ability to use dietary interventions to lessen the decline in immune function seen in older age would also be highly advantageous, as reduced immunity plays a large role in the development and progression of age-related disease (Makinodan and Kay, 1980). Immune function is not something that can be easily measured *in vivo,* though, as it is generally only activated in the presence of an invading pathogen. Certain assays have been devised where the immune response is assessed following an artificial immune challenge (Fang *et al.*, 2000; Akbulut *et al.*, 2001) but these were deemed to be inappropriate for this study as aging appears to have little effect on humoral immunity indices (*i.e.* B cell subpopulations and proliferation) (Lesourd, 1990a). Instead, the assessment of immune function used in this study focused on the simple measure of neutrophil function. Neutrophils mediate the immediate host response to bacterial and fungal infections, which are largely responsible for the higher rates of mortality and morbidity seen in older populations (Butcher *et al.*, 2001). In human populations, total neutrophil numbers are not thought to decline; however, a decrease in neutrophil function (phagocytic activity) has been shown to occur with increasing age (Mege *et al.*, 1988; Wenisch *et al.*, 2000; Butcher *et al.*, 2001).

In this study, the degree of neutrophil phagocytosis was similar in both honey- and sucrose-fed rats (75–80%), both of these treatments having approximately 50% higher levels of phagocytosis than rats fed the sugar-free diet. Many other studies have investigated neutrophil function in rats (including Wheeler *et al*., 1994; Chawla *et al*., 1995; Wise, 1996; Lin *et al*., 1996, Bober *et al*., 2000), but given that every trial used different diets, breeds, age and drug interventions it is impossible to get comparable data, or data that could be possibly be used as an age-equivalent baseline. Thus, it is impossible to know whether the higher levels of neutrophil phagocytosis in honey- and sucrose-fed rats reflect a reduced decline with age, or whether there has been a stimulation in phagocytic activity in these two groups that did not occur in those fed the sugar-free diet. A review of the literature revealed nothing that might suggest that sucrose could affect the activity of the immune system, although there is some limited data available for the effects of feeding honey on neutrophil counts. In one small study (Al-Waili, 2003b) 12 sheep (6–8 months; 25–30kg) were randomised to receive either 500 ml of intravenous honey (5%) or saline once every 10 days for 40 days, with blood removed at 0, 10, 20, 30, 40 and 50 days. The % neutrophils (it is not stated, but the reader must assume that the author mean the percent of leukocytes that were neutrophils) increased from 55% at time zero to

76% in the honey group after 10 days, whereas no difference was observed in the saline group. In the honey-fed sheep the % neutrophil levels remained elevated for 30 days before decreasing to baseline levels again on day 50. No data are available in that study as to what the level of neutrophil phagocytosis may have been in those animals, nor are the animals considered to be in old age, but is still of interest to note that the total number of neutrophils increased so dramatically. In the present study, no actual neutrophil counts were measured, but it is possible that the increased phagocytosis seen in honey- and sucrose-fed rats resulted from an increase in the number of new (and maybe more active) neutrophils. This idea is supported by the finding that the % of leukocytes that were lymphocytes was higher in honey- and sucrose-fed rats compared with those given the sugar-free diet (and also higher in honey-fed rats compared to those fed sucrose). Lymphocytes consist primarily of B cells, T cells and Natural Killer cells (Wenisch *et al.*, 2000) thus an elevated lymphocyte count could reflect an improvement to either or both of the cell-mediated and humoral immune responses. Honey has been shown to have a mitogenic effect on human B and T lymphocytes (Abuharfeil *et al.*, 1999) and it has been suggested that this may occur through the glycosylation of proteins resulting from the high sugar content (glycosylated proteins have been shown to activate a number of cell types; Brownlee, 1995).

As mentioned earlier in Chapter 7, sugars that are slowly absorbed can lead to the formation of short chain fatty acid (SCFA) fermentation products in the gut. SCFA are commonly formed after ingestion of pre-biotic foods, and with the pre-biotic effects of honey established *in vitro* (see Chapter 1) it is possible that the ingestion of honey may also result in their formation. A number of studies have demonstrated, either directly or indirectly, that SCFA can have immunomodulatory properties (reviewed by Schley and Field, 2002). Thus it is possible that honey may also be stimulating the immune system via the presence of fermentable sugars in the gut. Much more research is needed to elucidate if this is so.

It is also possible that non-sugar components of the honey may be responsible for the enhanced immuno-modulatory effects seen. Tonks *et al.* (2007), for example, have recently reported that a 5.8 kDa component of Manuka honey is capable of stimulating immune function *in vitro*. These authors determined that the compound was not a lipopolysaccharide, amino acid, vitamin or mineral, but further investigations are required to elucidate the nature of the compound. Furthermore, it is possible that the antioxidant content of the honey

may have contributed to its immuno-modulatory effects. Although there are no studies directly investigating the effects of honey antioxidants on immune function, other antioxidant compounds have been shown to stimulate immune function *in vitro* (Sabongi *et al.* 1997) and *in vivo* (De La Fuente *et al.* 2002).

Aging in human populations has also been associated with changes in the T cell subpopulations (Ales-Martinez *et al.*, 1988; Lesourd, 1990b; Cossarizza *et al.*, 1992; Lesourd and Meaume, 1994). Due to the costs of the antibodies involved, it was not possible to assess these lymphocyte subsets directly in this study; although it would certainly be of interest to assess whether honey could influence these subpopulations (or other aspects of cell-mediated immunity) in future work. Similarly, despite the evidence that humoral immune response probably does not alter significantly in older age, it would be advantageous to determine what role honey might have in improving humoral immunity.

Few differences between treatments were found in the bone-related DEXA measurements performed in this study, which lends support to the findings in Chapters 7 and 8 that honey does not improve bone calcium levels. Certainly, no differences in any of the DEXA endpoints were observed between rats fed sucrose and honey, suggesting that the type of sugars ingested long-term may have little impact on bone density or mineralisation levels. No other long-term data is available for the effects of honey on bone density measures, but evidence suggests that long-term feeding of high sucrose diets can alter the calcium balance in humans and negatively effect bone mineralisation levels (refer to Chapter 8). Such decreases in bone density and mineralisation were not seen in sucrose-fed rats in the present study, but it may be that the levels of sugars were not high enough to induce reductions in bone strength and density (calcium levels in the aforementioned studies were up to 65%). In addition, the addition of skim milk powder to the diets in this current study may have aided calcium absorption in all three dietary treatments due to the presence of casein and the possible formation of casein phosphopeptides in the gut.

The degree of oxidative damage was assessed in this study as a means for gaining some measure of whether the antioxidant content of the honey could offset some of the free-radical damage that occurs in older age. However, as with measurement of immune function, measurement of oxidative damage is fraught with difficulty. The usual methodologies for quantifying oxidative damage *in vivo* either measure DNA damage (via the presence of the oxidised base 8-hydroxy-2-deoxyguanosine (8OHdG)) or protein damage, specifically protein carbonyl formation (reviewed by Halliwell and Whiteman, 2004). However, whereas both

DNA damage (Richter *et al.*, 1988; Gedik *et al.*, 2005) and protein carbonyl formation (Buss *et al.*, 1997; Davies *et al.*, 2001) are simple to measure (using ELISA), neither method can accurately quantify the damage accumulating over the span of a lifetime: measurement of 8OHdG typically results in artificial oxidative damage arising during the DNA isolation process, and both protein and damaged DNA are turned over fairly rapidly inside the cell. Collagen molecules, in contrast, have an exceptionally long lifetime, which makes them good markers for accumulated oxidative damage. In particular, collagen is susceptible to the accumulation of AGE products which react to form fluorescent adducts. The level of MDE-protein adducts measured in the aortic collagen samples was significantly higher (~ 5%) in the sucrose-fed rats after 12 months compared with animals fed either the honey or sugar-free diets, and it is possible that this is due to differences in the glycemic indices of the diets. Odetti *et al.* (1996) and Traverso *et al.* (1998) have both demonstrated that age-related accumulation of AGE products is higher in rats with poor glycemic control and suggest that this is due to hyperglycemia-induced oxidative stress (the significance of oxidative stress is discussed further in Chapter 10). This certainly fits with the data presented in this study as animals fed of the low GI diets sugar-free diet had comparable lower levels of MDE fluorescence compared with those animals fed sucrose.

Interesting, it has been reported in a well designed study that rats fed isocaloric diets containing either cornstarch, glucose, sucrose, fructose or equimolar amounts of fructose and glucose as their carbohydrate source (660 g/kg) all exhibited similar levels of AGE formation in the skin, aortic, tracheal and tail tendon collagen at 9, 18 or 26 months of age (Lingelbach *et al.,* 2000). This data is interesting as the treatment group receiving the fructose:glucose combination provides a close comparison for the honey treatment group in this study. Obviously, direct comparisons cannot be drawn due to the different rats used and the fact that the sugar composition of honey is not as simple as a 1:1 fructose/glucose mixture, but it suggests that either the different ratio of these sugars in honey or the presence of other components of the honey (either minor sugars or non-sugars) induces a lower level of AGE formation.

It is unknown what effect(s) a small difference such as 5% could have in human health; however, given the role that oxidative stress can play in the development and progression of many age-related diseases, the findings are promising. Recent research by Schramm *et al.* (2003) has demonstrated that plasma antioxidant and reducing capacities increased significantly by 5–30% in

volunteers 1–6 hours after consuming honey (1.5 g/kg body weight). This is a fairly high level of honey ingestion (105–120g for a 70–80kg person) and certainly more than the average person would eat in a day (let alone in one sitting) but it does show that the antioxidant content of honey is bioavailable after consumption.

In conclusion, honey does appear to have a number of health benefits associated with long-term feeding. These appear to result from a number of different factors including its antioxidant content. A lot of the work undertaken in this trial was preliminary at best and restricted by budgetary constraints. However, it provides good evidence to suggest that honey may be a healthier replacement for sucrose, particularly in individuals who are overweight or at high-risk for cardiovascular heart disease. Of course, a crucial flaw of this study was not having the resources available to assess the glycemic index of the different diets to ascertain whether consumption of honey and sucrose resulted in similar or differing glycemic response. This information would certainly aid in understanding how honey and sucrose can have different effects on weight gain and biochemical measures, and it would be essential in any further studies in this area. The further roles of long-term feeding of honey are addressed in Chapter 10.

Chapter 10:

The Long-Term Feeding Effects of Honey versus Sucrose and a Sugar-Free Diet on Anxiety and Cognition in Rats

As mentioned at the start of Chapter 9, this current chapter presents the results from the behavioural measures (relating to the cognitive function) from the 12-month feeding study undertaken between February 2006 and February 2007 to determine whether there were any differential effects of feeding honey, sucrose or a sugar-free diet long-term. All the data pertaining to the non-behavioural measures (weight regulation, lipid profiles, bone densities *etc*) has been presented in Chapter 9.

The main findings of this chapter have been submitted for publication as follows:

Chepulis, L., Starkey, N., Waas, J., Molan, P. (2008) The effects of honey compared with sucrose and a sugar-free diet on anxiety and cognition during long-term feeding in rats. Submitted to the *Journal of Physiology and Behavior*.

10.1 INTRODUCTION

As discussed previously in Chapter 9, many aspects of health are known to deteriorate in older age. In both humans and animals, aging is associated with a slow deterioration of cognitive performance, and particularly of learning and memory (Ingram *et al.*, 1994; Grady and Craik, 2000). Cognitive decline or impairment not only deteriorates with increasing age though, but can also vary widely within a population (Awad *et al.*, 2004). Recently, evidence has suggested that deteriorations in learning and memory can occur during middle age rather than just in older age (Lamberty and Gower, 1990, 1992; Feeney *et al*., 2002), and that cognitive declines in middle age can predict deficits in these areas in later life (Lamberty and Gower, 1992; Stone *et al*., 1997).

Oxidative damage has long been proposed to be critically involved in the pathological manifestations of aging (see Section 9.1) including the decline in cognitive performance (Kumar and Gupta, 2002). However, the intracelllular oxidative balance is a tightly regulated system and an increase in oxidative stress generally results in an upregulation of antioxidant compensatory mechanisms including the induction of Cu/Zn superoxide dismutase, catalase, glutathione reductase, peroxiredoxins and a number of heat shock proteins (Berr *et al.*, 2004; Guidi *et al.*, 2006). It appears, however, that endogenous antioxidant defenses are often insufficient in older age, they being unable to detoxify all the oxygen free radicals that are continually being generated. The end result is oxidative damage to critical biological molecules (including DNA, protein and membrane lipids) leading to age-related neuronal loss and/or dysfunction (Harman, 1992).

The production of free radicals (as a side product during the reduction of molecular oxygen) is thought to be higher in cerebral tissue than in other tissues, with this tissue being more susceptible to damage, because of its low content of antioxidants, high content of polyunsaturated fatty acids in neuronal membranes and high oxygen requirements for metabolic processes (Guidi *et al.*, 2006). Research has demonstrated that there is a progressive increase in the steady state concentration of oxidatively-modified DNA and proteins in the brain during aging in both animals (Carney *et al.*, 1991; Leutner *et al.*, 2001) and humans

(reviewed by Forster *et al.*, 1996). As well, a large clinical study has shown that there was a substantial reduction in glutathione peroxidase levels in elderly individuals who demonstrated a decline in cognitive performance (Berr *et al.*, 2004).

Evidence for the involvement of oxidative stress in age-related dementia and cognitive decline has also come from research investigating the causes of severe cognitive impairment disorders such as Alzheimer's disease. Increasing evidence supports a role of oxidative stress in the development of this disease, as an increase in oxidative metabolites of DNA, lipid and protein have been observed in the blood and post-mortem brain samples of Alzheimer's subjects compared with healthy individuals (Guidi *et al.*, 2006). In addition, a number of studies have reported that there are direct correlations between increases in oxidative damage with reductions in learning (Vishnevskaya *et al.*, 1991; Liu *et al*., 2003; Jhoo *et al*., 2004; Wu *et al*., 2004) and memory (Fukui *et al*., 2001; Jhoo *et al*., 2004; Pieta Dias, 2007).

Importantly, researchers are now becoming aware that there are strong links between both impaired glucose tolerance and type II Diabetes with cognitive function. A large number of studies have examined the impact of type II Diabetes on brain function and dementia (reviewed by Messier, 2005), and the general conclusion is that type II Diabetes is associated with an increased incidence of cognitive impairment, particularly in those older than 70 years, and those with concomitant cerebrovascular disease and hypertension. Several studies have also shown that middle-aged subjects with Diabetes are at a higher risk of developing dementia and cognitive decline (Di Bonito *et al.*, 2007), raising the idea that the metabolic abnormalities of Diabetes such as hyperglycemia and insulin resistance may play a role in the development of cognitive decline in elderly subjects. Indeed, a number of studies have shown that impaired glucose tolerance has a significant impact on working memory, verbal declarative memory and executive functions (reviewed by Awad *et al.*, 2004). Similarly, Di Bonito *et al.* (2007) have demonstrated that impaired fasting glucose levels, hyperhomocysteinaemia and insulin resistance are strongly correlated with impaired cognitive function in elderly non-diabetic subjects.

Hyperglycemia may directly damage the brain through a number of mechanisms, the most likely being an increase in the generation of oxygen free radicals and the formation of AGEs (both of which have been associated with Alzheimer's disease) (Vitek 2004). The adverse effects of high blood glucose on cognitive performance have been confirmed by studies showing that improving

glycemic control improves cognitive abilities in older people with Diabetes. Similarly, a limited number of studies have also demonstrated that dietary GI can have an effect on cognitive performance (because of hyperglycemia-induced oxidative damage). In one small study involving elderly diabetic subjects (mean age 65 years), cognitive performance was significantly improved after short term consumption of a low-GI diet compared with a high-GI diet (Papanikolaou *et al.*, 2006). A similar finding was also observed in younger subjects (mean age 21 years) (Benton *et al.*, 2003) however, the effects of long-term feeding of a low *vs* high GI diet on cognitive performance appear to have not been investigated.

Guidi *et al.* (2006) have shown that production of reactive oxygen species is correlated with age but not specific dementia disorders, and suggest that the oxidative imbalance observed in mild to severe cognitive decline is due to a decrease in total antioxidant capacity rather than an increase in reactive oxygen species. For this reason, research is now focusing on the availability of dietary antioxidants, and the use of supplemental antioxidants as a way of reducing the severity of cognitive decline.

Animal studies have demonstrated that oxidative damage can be attenuated relatively rapidly in older animals following the consumption of certain antioxidant compounds (Carney *et al.*, 2001; Kolosova *et al.*, 2006a). The antioxidant phenyl-α-*tert*-butly-nitrone, for example, has been shown to reduce the amount of brain protein oxidation and improve cognitive performance after both short- and long-term feeding (Carney *et al.*, 1991). In addition, cognitive improvements were observed in aged rats (80 weeks old) but not in young rats (8 weeks old) fed extracts of the plant *Equisetum arvense* L that has antioxidant properties (Guilherme dos Santos *et al.*, 2005). Similarly, aged dogs fed a diet supplemented with vitamins E and C and/or a mixture of fruit and vegetables for 6 months had significantly improved cognitive abilities compared with those fed standard chow (Cotman *et al.*, 2002; Ikeda-Douglas *et al.*, 2004). Furthermore, a number of studies have specifically demonstrated that spatial and recognition memory (Carney *et al.*, 1991; Dairam *et al.*, 2007; Pieta Dias, 2007) and learning (Liu *et al.*, 2002, 2003; Ying and Gomez-Pinella, 2004; Duffy *et al.*, 2007) can be enhanced in older animals after feeding substances that reduce oxidative damage. Thus, there appears to be strong evidence to suggest that antioxidant intake may help attenuate the reductions in memory and learning seen in middle- and older-age.

In large well-designed clinical studies, however, the effects of antioxidant intake on cognitive performance have been equivocal. Peacock *et al.* (2000)

demonstrated that cognitive performance in middle-aged adults (mean age 56 years) was in no way correlated with dietary antioxidant intake or supplemental use. Similarly, in the Zutphen Elderly Study, neither β-carotene nor vitamins C or E had any effect on cognitive impairment or decline in elderly men aged 69–89 (Kalmijn *et al.*, 1997), and no beneficial effects of vitamin C and E or β-carotene on cognitive abilities were observed in elderly subjects (mean age 75 years) when given as an antioxidant supplement for nearly 7 years (Yaffe *et al.*, 2004).

However, the Rotterdam Study (mean age 68 years) demonstrated that a lower dietary intake of β-carotene (but not Vitamins C or E) was associated with impaired cognitive performance (Jama *et al.*, 1996), and the Cache County Study reported that β-carotene and Vitamins C or E may delay cognitive decline in the elderly (Wengreen *et al.*, 2007). Again, though, there appears to be no literature published on the long-term use of either dietary or supplemental antioxidants on the possible reduction of cognitive decline in later life. However, data from clinical and epidemiological studies does suggest that antioxidants may reduce the risk for cerebrovascular disease in later life (Gey *et al.*, 1993; Rimm *et al.*, 1993; Kardinaal *et al.*, 1993; Jama *et al.*, 1996) thus this area of research is still being studied extensively.

Because of concerns about the safety of synthetic antioxidants, many studies have focused on the beneficial effects of natural sources of antioxidants, especially those derived from fruits and vegetables. Berries, in particular, have been a popular area of research in recent years, as they have been demonstrated to contain high levels of natural antioxidants. In addition, a few studies have shown that berry-enhanced diets can improve cerebellar physiology and cognitive performance in both animals and humans (Bickford *et al.*, 2000, Lau *et al.*, 2005; Ramirez *et al.*, 2005; Ramassay, 2006). Honey has also been shown to have strong antioxidant properties *in vitro* (refer to Section 1.4), the darker coloured honeys generally having higher antioxidant levels (Gheldof and Engelseth, 2002). In addition, intake of antioxidant-rich honey has been shown to increase serum antioxidant levels in humans (Al-Waili, 2003c; Gheldof *et al.*, 2003; Schramm *et al.*, 2003) and to reduce lipid peroxidation in cooked poultry (Antony *et al.*, 2000; McKibben and Engelseth, 2002). The long-term use of honey as an antioxidant has never been investigated though, thus its potential ability to reduce cognitive impairment in older subjects has never been fully explored.

The aim of this study was therefore to assess the cognitive abilities of rats during long-term feeding of either honey, sucrose or a sugar-free diet. A number of cognitive measures were assessed at set points throughout the 12-month duration of the study to determine whether there was a difference in the rate of cognitive decline between the three diet groups, whilst other measures were assessed only at the completion of the study.

10.1.1 Choice of Behavioral Tasks

There are many behavioural and ethological methodologies that can be used to assess cognitive functions in animals; however, they can differ widely depending on whether the animals require training prior to performing a task, whether the task is a stand-alone event or a repetitive exercise, how the animals are supposed to learn a given task and what reward system is used.

Most behavioural tasks use some sort of food reward system as a means by which the animals are enticed to do a particular task, with the animals usually reduced to 80–85% of their pre-testing body weight (Komaki, 2004). Given, however, that assessment of cognitive ability was only one facet of the experiment in which these rats were fed on the different diets long-term, and that weight gain and food intake were key endpoints in another aspect of this experiment (refer to Chapter 9), depriving the animals of food and providing food rewards was not feasible in this study. Therefore, only tasks that used other means of positive reward or negative consequence were employed.

As well as there being many tasks available to assess animal behaviour, there are also many aspects of cognition that can potentially be investigated. However, due to time and financial constraints in this study, only a limited number of tasks to assess cognitive abilities could be set up for this trial. The maze tasks for this study were therefore chosen based on their reported effectiveness in the literature, their relatively low set-up costs and the areas of cognition that they potentially assessed. The intention was to cover as many aspects of cognition as possible. Initially the areas of cognition that were to be assessed were anxiety (using an Elevated Plus maze), spatial learning/recognition memory (using a Y maze) and active learning (using passive avoidance). Measurement of passive avoidance, using methods from the literature that used white noise (Halladay *et al.*, 2000; Ledgerwood *et al.*, 2005) or a mild electric foot shock (Halladay *et al.*, 2000; Torres *et al.*, 2005; Schneider *et al.*, 2006) as a negative stimulus proved difficult in initial testing though and were abandoned before the first testing session. Additional tasks were then designed and included in the study after the

trial had begun. A decision was made to assess exploratory behaviour (using an Open Field task) as the degree of movement that an animal exhibits may be a confounding factor in its level of performance in other tasks. An Object Recognition task was also added to the behavioural suite of assessments as recognition memory is one of the key areas of cognition that is known to deteriorate with age (Berr, 2002). A second learning task where animals learned to turn off a bright light was also investigated, and used later in the study to determine if the learning aspects of cognition could be influenced by the experimental diets. A brief overview of the rationale behind each task is given in the relevant sub-section in Section 10.2 (see below).

10.2 MATERIALS AND METHODS

10.2.1 Experimental Animals, Housing and Diets

All experimental details related to the dietary aspects of the design of this trial and the maintenance of the rats are given in Section 9.2. The composition of the experimental diets is given in Table 9.1.

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10.2.2 Behavioural Studies: Overall Design

All maze tasks were carried out in a separate room of the experimental facility where the animals were housed. As with the room in which the animals were housed, the temperature of the testing room was maintained at approximately 22^{o}C. With the exception of the Y maze, which required two phases of testing, all tasks were carried out between the hours of 0800–1200. This was the start of the night time phase for the animals (lights were off at 0630) when they should be most alert and active. The lights were kept switched off in the testing room and in the hallway throughout the entire duration of testing. A 40 watt lamp was turned on in the corner of the testing room and positioned so that it was facing the wall near the floor. This provided minimal light in the testing room (maximum 40 lux) allowing the animals to see cues on the walls of the maze and the room. A similar lamp was turned on in the room in which they were housed 30 minutes prior to testing and for the duration of testing to allow them to acclimatise to the light level before being moved to the testing room.

Unless specified otherwise, all animals in each diet group performed every task. The order in which the animals performed each task was randomised prior to beginning the first test, and they were placed into each maze according to this order (no observers were present in the testing room other than to place the rat in the maze and collect it upon completion). All maze work was videotaped

from above and from different angles (1-3 cameras depending on the task) and the videotapes scored within one month of the test sessions using Hindsight software (Version 1.5, designed by Scott Weiss, University of Leeds). The videotapes were scored blind using the same random ordering and the session data was not collated back into dietary groupings until all animals in a given session had been scored.

Unfortunately due to the time that it took to have the mazes made, no baseline behavioural measurements were able to be taken. Unless reported otherwise, the tests were commenced 3 months into the study (at 5 months of age) and performed every 3 months for the duration of the trial.

10.2.3 Elevated Plus Maze

The elevated plus maze (EPM) is a simple method for the measurement of anxiety and it has been used widely in the literature with good results (reviewed by Kulkarni and Sharma, 1991; Rodgers and Dalvi, 1997; Carobrez and Bertoglio, 2005). This task was chosen because whilst anxiety is not directly linked with cognitive decline, there is evidence to suggest that anxiety often presents with depression, and that both anxiety and depression may occur to a greater extent in individuals with cognitive disorders (Dupuy and Ladouceur, 2007; Herrmann *et al.*, 2007; Mantella *et al.*, 2007; Steffens and Potter, 2007).

The EPM task works on the theory that rats have an innate tendency to explore new environments but also a fear of open spaces. Importantly, this task does not require the animal to learn the task prior to undertaking it, and the influence of non-specific factors such as those generated by aversive or food reinforcements is not involved. As the EPM is an anxiety test, it was always the first task performed in each testing period (*i.e.* before the animals became used to being out of their cages).

10.2.3.1 Apparatus

The design of the EPM was based upon measurements given by Sayin *et al.* (2004). The maze consisted of two open arms that were 50 x 10 cm at right angles to two closed arms that were 50 x 40 x 10 cm (see Figure 10.1). The open-sided central area of the maze was 10 cm square (see Figure 10.1). The maze was made out of dark grey PVC plastic and painted with matt black paint on the insides of the closed arms and the top of the open arms. White lines marked out the centre area so that it was clearly visible on the videotape when the animal moved into a new area of the maze. The maze was elevated to 50 cm

above the ground to provide an aversive stimulus to animals in the open arms, preventing them from jumping off the maze.

Figure 10.1: *Representation of the elevated plus maze*.

10.2.3.2 Testing Routine

Each animal was placed in the centre area of the maze, with its head facing towards an open arm. The rat was then allowed to explore the EPM for 5 minutes before it was taken back to its home cage. The maze was fully cleaned with mild detergent between animals.

Based upon conventional parameters of Rodgers and Johnson (1998) and Cole and Rodgers (1995), activity within the EPM were determined by scoring a pre-defined set of behavioural activities (see Table 10.1). Once scoring was complete, the following behavioural measures were calculated: total arm entries, open and closed arm entries, percentage of open and closed arm entries, duration open and closed arm time, duration of centre square time, frequency of rearing, latency to enter the open arm, frequency of protected and unprotected head dips, frequency of stretch attend and frequency of half body out. In the event that an animal fell off the EPM, it was not placed back onto the maze, although it was recorded as having not completed the task. Data from any animals that fell were excluded from the analyses.

Table 10.1: *Definition of behavioural elements in the elevated plus maze*

Behaviour	Description / Definition
Arm Entry	All four paws in an arm
Rear	Front paws off the floor, with the body stretched upward, either supported by the maze wall, or unsupported in the open arms
Head Dip	Exploratory action, where head and shoulders are placed over the side of the maze, with the animal looking towards the floor. The head dip was deemed to be unprotected if the animal's body was in the open arm at the time, and protected if the animal's body was in either the closed arm or centre area.
Stretch Attend	Exploratory movement in which the animal stretches forward (often peering into the open arm from the closed arm) then returning to the original position
Half Body Out	Exploratory movement where the animal is in the centre square and moves the front half to two-thirds of its body out into an open arm. Front paws are in the open arm, whilst back paws remain in the centre square

10.2.4 Y Maze

The Y maze task is designed to assess recognition memory and spatial learning. It has been used successfully in a number of other studies, some of which used additional reinforcements (*eg.* food, electric shock) (Cazala and Zielinksi, 1983; Rao *et al.,* 2001 Holden *et al.*, 2004; Levshina and Shuikin, 2004), whilst others did not (Conrad *et al.,* 1996, 1997, 2003; Taghzouti *et al.* 1999). This task was chosen because of its relative ease of use, and because recognition memory is well accepted as being one of the key areas of cognition that declines in older age (Verhaeghen *et al.,* 1998; Clarys *et al.,* 2002; Prull *et al.,* 2006; Gutchess *et al.,* 2007).

The Y mazes task is designed to use a rat's innate desire to explore new environments (Granon *et al.,* 1996), thereby assessing memory and learning by

determining whether the animal can remember which arms of the maze it has visited previously, and whether it can spatially orient itself appropriately so as to visit the unvisited arms of the maze when placed into it on a second occasion. Further details are given below.

10.2.4.1 Apparatus

The design of the Y maze was based primarily on that of Conrad *et al.,* (1997) and Taghzouti *et al.* (1999). The maze consisted of three closed arms that were each 50 x 16 x 32 cm high and all 120^{o} apart (see Figure 10.2). The maze was made out of grey PVC plastic and painted matt black on the inside. A layer of mixed soiled bedding (wood shavings *etc*) was placed in the Y maze to mask any olfactory cues from previous animals. These shavings were mixed thoroughly, and the walls of the maze wiped down with mild detergent after every animal. A removable door made from the same PVC plastic as the maze was used to block access to one arm as required (see Figure 10.2). Spatial clues were affixed to the end of each arm (cardboard 15 x 15 cm with an image of either a black triangle, a patterned circle or a patterned square) and on the walls of the testing room (at a height that allowed them to be seen over the walls of the maze).

10.2.4.2 Testing Routine

Y maze testing consisted of two phases. During Phase 1 the animal was placed into the end of one arm (facing the centre) and allowed access to that arm (the START arm) and one other (the OTHER arm) for 10 minutes. The third arm (the NOVEL arm) was blocked by the removable door. Choice of which arm was 'start', 'other' and 'novel' was rotated with each new animal, and all the rats were randomised for the sequence of placement into the maze. The rat was then removed from the maze and returned to its home cage in the housing room for an inter-trial duration of 4 hours as previous research has shown that Sprague Dawley rats demonstrate no recognition memory after 6 hours but that recognition memory occurs for time periods of 4 hours or less (Conrad *et al.*, 1996; Taghzouti *et al.*, 1999).

After 4 hours had lapsed the rat was placed back into the start arm of the maze and given free access to all the three arms for five minutes (Phase 2). The arm they first chose to explore after initially leaving the 'start' arm was recorded, as well the latency to enter the 'novel' arm and the number and duration of explorations of each arm and the centre area. These latter data were recorded each minute over the five minute period. During both phases, entry into (and

duration in) a particular arm was defined as placement of all four feet at least 5 cm into the arm. Animals were excluded from the analyses if they did not leave the start arm for the duration of the testing session, and if they spent >75% of their time in the centre area during a given time interval.

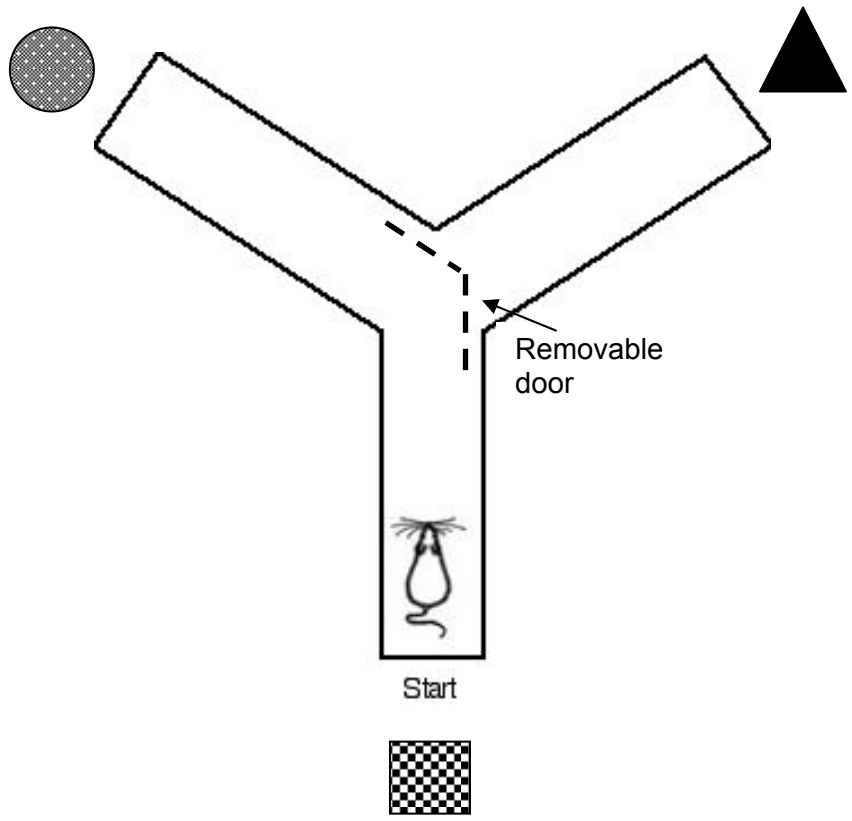

Figure 10.2: *Diagrammatic representation of the Y maze showing the removable door and the objects used as spatial cues.*

10.2.5 Open Field Study

An Open Field task was undertaken at 6 months to assess locomotor activity across the three diet group groups as some animals showed only minimal movement in the Y maze task and it was unknown whether this was due to dietary influences or other factors. The task was again undertaken at 12 months to determine if diet had any affect on levels of locomotor activity in older animals. The Open Field task was based upon the work of Iuvone *et al.*, (1996), Stohr *et al.*, (1998) and Bowman *et al.*, (2002), and simply assesses the degree of movement within an arena over a given period of time.

10.2.5.1 Apparatus

Large Perspex sheets were covered in black polythene and joined together to form the four 50 cm high walls of an enclosed arena that was 100 x 100 cm. The arena was placed onto the floor of the testing room, with a thick layer of soiled bedding covering the floor of the Open Field maze. The soiled bedding was randomly mixed around between animals. On the monitor used for scoring a sheet of clear plastic was overlaid on the image of the arena, and the area divided into a 5 x 5 grid, each section of the grid being 20 x 20 cm (this area being greater than the head to rump lengths of all animals). Using this grid, the arena was divided into three zones: centre, middle and outside (refer Figure 10.3).

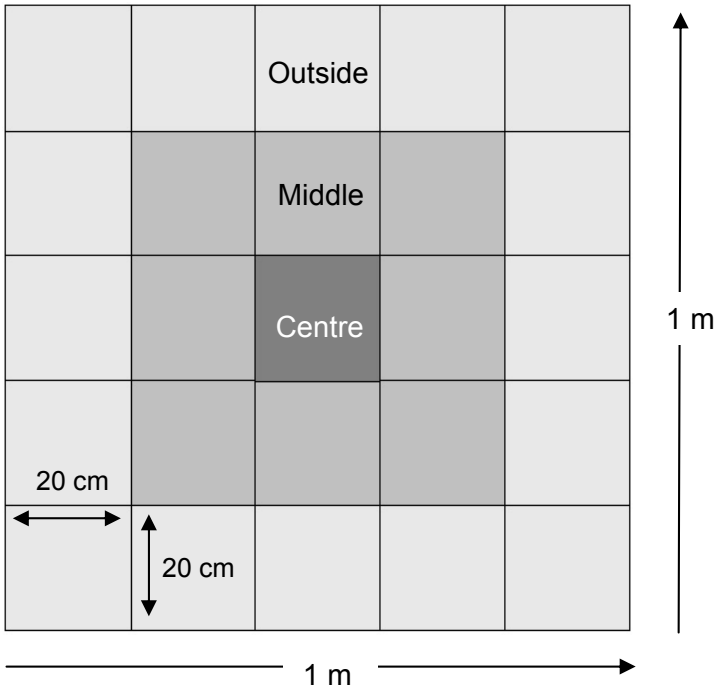

Figure 10.3: *Diagrammatic representation of the Open Field arena showing the centre, middle and outside zones.*

10.2.5.2 Testing Routine

Each animal was taken in its home cage from the housing room to the testing room. At the beginning of the test the animal was removed from its cage and placed in the centre zone of the Open Field arena (see Figure 10.3). The rat was then given 5 minutes to explore the open area and all movements recorded. The animal was then placed back into its home cage in the testing room, and the

cage kept in that room for the Object Recognition task (see Section 10.2.6). All animals were tested and scored in a random order.

Once scoring was complete, three time bins were chosen for analysis: the full 300 seconds, and 0-150 and 151-300 seconds to determine if the rate of locomotor activity altered during the first and second half of the testing period. The following behavioural measures were then calculated for each time bin: duration in centre, middle and outside zones, frequency of entries to each zone, number of line crosses within the middle and outside zones, and the total number of movements (including zone entries and line crosses).

10.2.6 Object Recognition Task

To determine if the different diets could affect object recognition learning in aged rats, an Object Recognition task was performed at the 12 month testing session. The methodology used was based upon the work of Ennaceur *et al.* (1997, 2005) and Andrews (1995) with modifications as described. This task was primarily included in the suite of behavioural assessments as other research has demonstrated that recognition memory declines in older age in both animals (Shamy *et al.,* 2006; Pieta Dias *et al.,* 2007; Wallace *et al.,* 2007) and humans (Rose et al., 1986; Murphy *et al.,* 1997), and that antioxidants may be able to offset this decline (Goyarzu *et al.*, 2004; Andres-Lacueva *et al.*, 2005). Furthermore, this task was a simple and inexpensive measure by which another facet of memory could be easily assessed.

In the research of the aforementioned authors, the Object Recognition task was a three-phase test with various inter-phase time periods. Phase 1 was a habituation phase that varied from one ten-minute session the day before testing (Andrews, 1993) to 4 ten-minute sessions over four days (Ennaceur *et al.*, 1997). Phases 2 and 3 were the experimental periods of the experiment, and these were generally the same in all three studies (see below for methodology). As time was limited at the 12 month testing period and both the Object Recognition task and the Open Field task use the same maze arena, the Object Recognition task was run immediately after the Open Field task above, with the 5-minute Open Field testing period also counting as the habituation phase of the Object Recognition task (see below). Only 10 animals per diet group (chosen at random) performed this task.

10.2.6.1 Testing Routine

After completion of the Open Field task, the animal was placed back into its home cage for 5 minutes and the cage ¾ covered with a black cloth to prevent the animals seeing where the objects were placed in the arena. During this 5-minute interval, two identical objects (A1 and A2 – see Figure 10.4a) were placed in the arena, each 10 cm away from a corner (two corners of one side of the arena were used). The rat was then taken from its home cage and placed back into the middle of the arena facing the test objects. The animal was given 3 minutes to explore the two objects (Phase 2).

After completion of Phase 2, the animal was again removed from the arena and placed back into its home cage in the testing room. During this 3-minute retention interval, the two objects were removed and replaced with an identical copy of those used in Phase 2 (A3) and a novel object (see Figure 10.4b) (Phase 3). These two objects were placed in the same locations as the previous objects. The animal was then placed back into the arena as described previously and allowed a further 3 minutes to explore the two objects. The spray bottle used as a novel object had been filled with water and the spray trigger disabled. The white containers were filled with small stones to prevent them from being tipped over.

Based upon the work of Ennaceur *et al.* (1997, 2005) a rat was considered to be exploring or visiting an object when its nose was either touching or < 2 cm away from the object. It was not considered to be exploring the object if it was sitting on it. The location (left or right) of the novel object was alternated with each new animal and all four objects were wiped down with mild cleaning solutions between each test.

The following behavioural measures were then scored and calculated: number of visits to object 1 or object 2 (Phase 2) and object 3 or novel object (Phase 3), duration of time spent exploring object 1 or object 2 (Phase 2) and object 3 or novel object (Phase 3), total time exploring either object and the latency (time taken to first explore) the novel object (Phase 3). Animals were excluded from the analysis if they did not explore either of the two objects in a given testing phase, and excluded from the latency data if they did not explore that particular object.

Figure 10.4: *The objects used in the Object Recognition task. In Phase 2 (3 minutes), animals had access to two identical objects (A). These objects were then removed and replaced with one identical copy of that used in Phase 2 and a novel object (B, Phase 3).*

10.2.7 Light Extinction Task

The ability to learn new tasks is also known to deteriorate with age (Savage and Bolton, 1968; Collie *et al.*, 2002; Royall *et al.*, 2003), although it is unknown how much of this may be influenced by diet. A simple task was therefore employed to assess the learning ability of rats fed the three different diets. The Light Extinction task used in this study is based upon the work published by Messaoudi *et al.* (1996) with modifications as described by Benton *et al.* (2003). In brief, the test is designed as a one-time experiment (*i.e.* they do not learn via repeated exposures) where animals have access to two levers, one of which turns off a bright light (active lever), the other of which has no effect (inactive lever). The measure of learning is whether the animals can differentiate between the two levers and keep the lights turned off (rats prefer dark environments). This task was performed at the 12 month testing session only.

10.2.7.1 Apparatus

The apparatus consisted of a standard rat cage (the same as those which they were routinely housed in) with two 75 watt angle-poise lamps positioned directly above the wire mesh top of the cage. Both lamps were plugged into a multi board and controlled via one switch. When illuminated, the light level in the cage was approximately 1200 lux. Two standard small animal levers with thin (8 mm) metal lever arms were modified by affixing pieces of thick plastic (5 cm x 3 cm) onto the thin metal arms with cellotape as initial testing demonstrated that the rats would not depress the thin levers (see Figure 10.5).

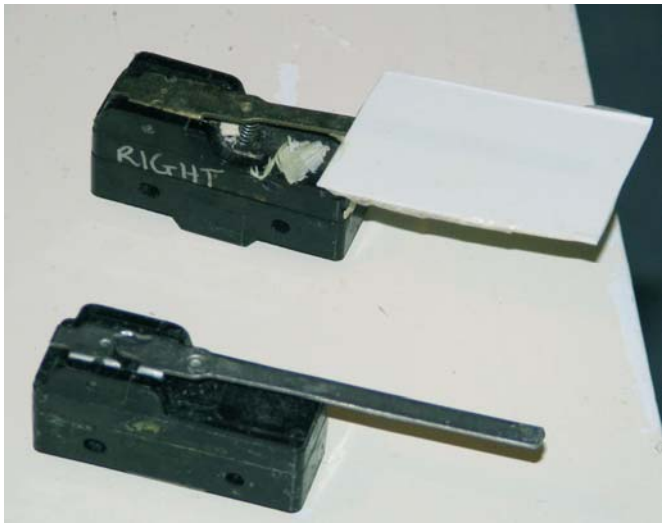

***Figure 10.5**: A modified (top) and un-modified (bottom) lever as used in the light extinction experiment.*

The levers were then affixed onto upside down plastic boxes and secured into the bottom of the cage (one at either end of the cage) (see Figure 10.6). A mixture of soiled shavings from various animals was placed in the bottom of the cage and mixed thoroughly between animals. A camera was attached to a nearby wall bracket, and the whole apparatus (including the lamps) covered with two layers of dark cloth. This ensured that light did not escape from the testing apparatus while the lights were on, and that no external light entered while the lights were off. An infrared camera was used to ensure that the rats could be observed when the lights were off.

Figure 10.6 *A standard rat cage, fitted out for use in the light extinction experiment. One lever was assigned to be the active lever (turning the lights off when pressed), the other the inactive lever (having no effect when pressed). Which lever was the active lever and which was the inactive lever was alternated with each new animal tested).*

10.2.7.2 Testing Routine

Ten rats, randomly selected from each diet group, were tested in random order in the Light Extinction task (the remaining animals were used in pilot trials to determine what size lever to use). Each animal performed one 20-minute session. At the start of the test, the rat was placed into the middle of the cage, with the lights off. The lights were then immediately turned on and the timer started. One lever was allocated to be the active lever throughout the session, whilst the other was the inactive lever. Which lever was the active or inactive lever was alternated with every new subject.

When the active lever was pressed, the lights above the cage were manually switched off for 30 seconds (using a switch on the multi-board) allowing for total darkness (no other lights were on in the testing room). Pressing the inactive lever had no effect; likewise pressing the active lever during the dark period did not prolong the period of darkness. All lever presses were recorded manually by watching the recorded sessions on videotape after all animals had completed the task. The number of active lever (AL) and inactive lever (IL) presses during periods of illumination (AL_{ON} and IL_{ON}) and darkness (AL_{OFF} and IL_{OFF}) and for both periods (AL_{TOTAL} and IL_{TOTAL}) were then determined for both the total 20 minute session and the first and second 10 minute periods.

Discrimination between the active and inactive levers whilst the lights were on was determined for the entire 20 minute testing session, as well as for the first and second 10 minute intervals. Learning was deemed to have occurred if the number of active lever presses was significantly higher than the number of inactive presses (*i.e.* if the AL/IL ratio was significantly greater than 0.5) as this suggests that the animal has learnt that pressing the active light extinguishes the light (rather than the active lever presses just being random events).

10.2.8 Statistical Analyses

Repeated-measure ANOVAs were performed on the EPM, Y maze and Open Field data to assess whether there were any interactions of diet with time. One-way ANOVAs were then performed on data from given time-points, or for particular measures where the repeated-measure analyses indicated a statistically significant difference. One-way ANOVAs were also performed on the Object Recognition and Light Extinction data to determine if there were any differences due to diet. As well, a mixed ANOVA was conducted on the Light Extinction data to compare the number of active and inactive lever presses across the three diet groups. Where ANOVA was significant, post-hoc tests using a Bonferroni correction were carried out to determine which groups differed. Chi Square tests was used to determine whether there was a statistical difference in the choice of arm entered first (other or novel) in the Y maze task, and whether the arm of first choice differed between dietary diet groups at a given time point. Paired t-tests were used to ascertain whether the number of active and inactive lever presses in a given diet group in the Light Extinction task were statistically different. All analyses were performed using SPSS version 12.0. (Note that presentation of the statistical findings in this chapter follow standard behavioural convention (*e.g.* F value (degrees of freedom); p value) and so differ from previous chapters).

The number of animals assessed in repeated-measures analysis often differs from the number of animals in each diet group due to the fact that not all animals completed each task at each time-point (fewer animals were generally tested at the 12-month testing session, for example, due to the need to perform additional tasks during that testing session) and that the *same* animals must be involved in each testing session for a repeated-measures analysis to be valid. In addition, if an animal was excluded from a task at a given time-point due to it not completing the task properly (*e.g.* falling off the maze) or because it died, the data from that animal was removed from the entire repeated-measures analysis.

10.3 RESULTS

As mentioned in Section 9.3, two animals were removed from the study in the first few weeks due to them being female, and the initial sample size for the remainder of the trial was 14, 14 and 15, respectively, for sugar-free-, sucrose- and honey-fed rats. In addition, two animals died later in the study, with a reduction in the sample size to 13, 14 and 14, respectively, for rats fed the sugar-free, sucrose and honey-based diets. With the exception of the two rats that died, all animals remained healthy throughout the 12-month period and no obvious illnesses were observed.

10.3.1 Elevated Plus Maze

The one major issue noted with the EPM task was that several of the rats had difficulty fitting into the closed arms of the EPM at the 12-month testing session due to their large size. Because of this, the 12-month EPM data was not included in the statistical analyses of this data, although full EPM Data from each testing session (including the 12-month data) are given in Appendix 2.

A repeated-measures analysis (n = 10, 11 and 14 for rats fed diets that were sugar-free or contained sucrose or honey, respectively) demonstrated that there was a significant effect of time ($F(2,64) = 8.5$; $p < 0.0001$), irrespective of diet, but that no diet *x* time interaction occurred. Overall, the mean duration of time spent in the open arms of the maze increased from 3.6 seconds at 3 months to 39.7 seconds at 9 months ($F(2,64) = 27.2$; $p < 0.0001$), this suggesting that all animals were less anxious as they got older (see Figure 10.7). In addition, the total number of rearing events (in the open or closed arms and the centre area) decreased significantly with time ($F(2,64) = 23.5$; $p < 0.0001$), with no effect of diet observed. Figure 10.8 shows the amount of time spent in the three areas of the maze at each time point for each diet group.

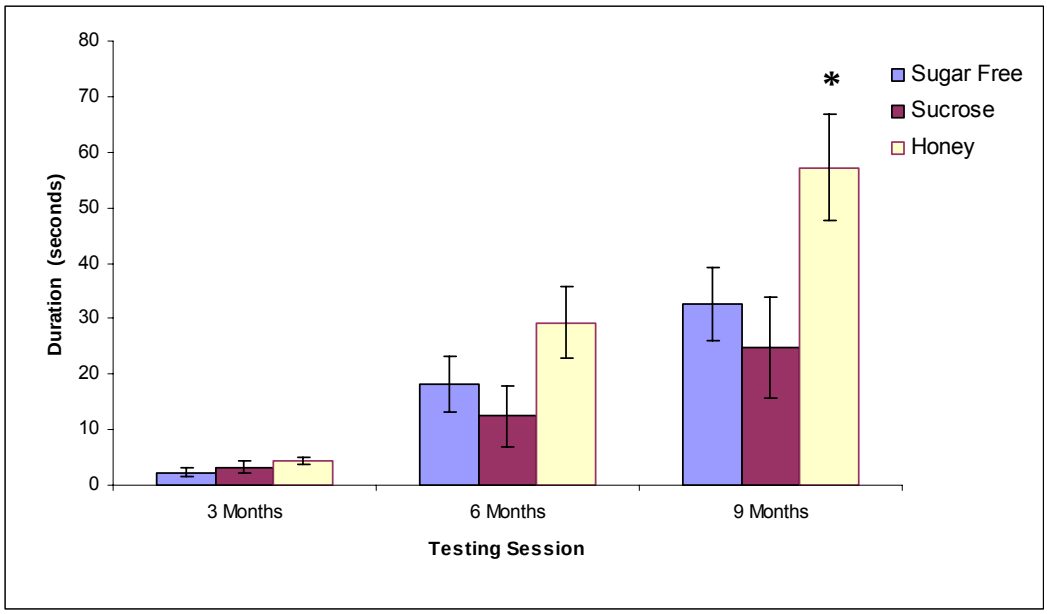

Figure 10.7: *Mean duration of time (seconds, ± SE) spent in the open arms of the Elevated Plus Maze for animals that were fed a diet that was either sugar-free (n=10) or contained 8% sucrose (n=11) or 10% honey (n=14). * significantly different to sucrose-fed rats at 9 months, p = 0.017*

The analysis also revealed a significant effect of diet, irrespective of time. The overall mean percentage of time spent in the open arms of the EPM was significantly higher in rats fed honey (13.7%) than in those fed a sugar-free diet (7.1%; $F(2,32) = 4.8$; $p < 0.05$). The overall mean percentage of time that sucrose-fed rats spent in the open arms of the EPM (7.8%) was similar to that of rats fed the sugar-free diet; however, the difference between sucrose- and honey-fed rats only approached statistical significance ($p = 0.056$). Similarly, the overall mean duration of time that was spent in the open arms of the EPM was significantly higher in honey-fed rats compared with those fed sucrose (29.3 *vs* 14.1 seconds; $F(2,32) = 5.0$; $p < 0.05$). Rats fed sucrose and the sugar-free diets were again similar (14.1 and 16.3 seconds) although the difference between honey-fed rats and those given the sugar-free diet did not reach statistical significance. (Note: for both these measures, animals not entering the open arm were included, but were given a value of 0 seconds). Figure 10.8 shows the percentage of time spent in the open arms of the EPM for all three diet groups at 3, 6 and 9 months.

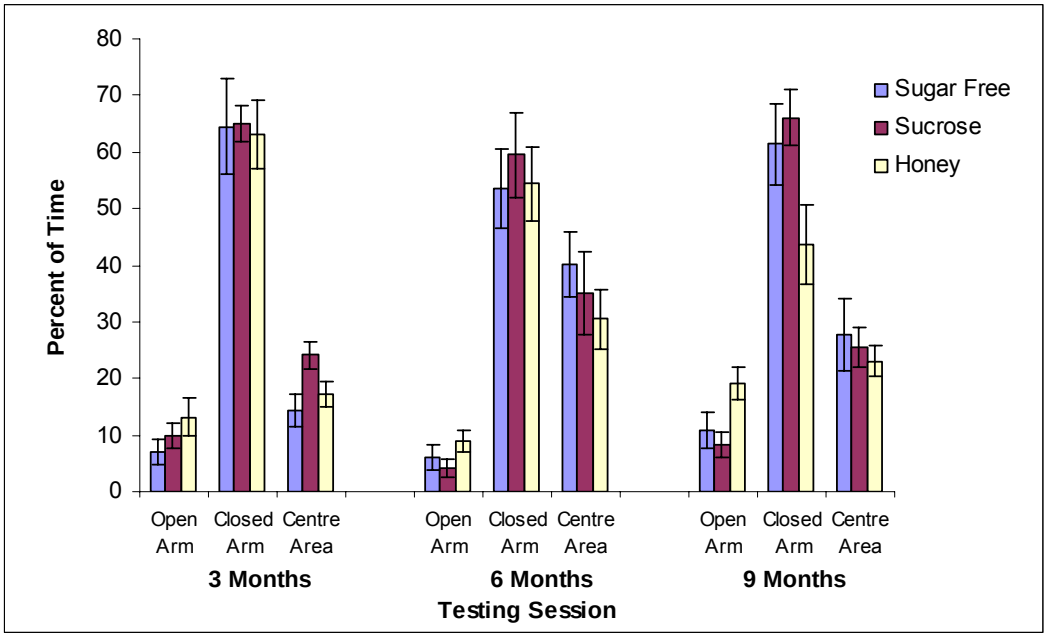

Figure 10.8: *Mean percentage of time (seconds, ± SE) spent in the open and closed arms and the centre area of the Elevated Plus Maze at 3, 6 and 9 months. Animals were fed diets that were either sugar-free (n=10) or contained 8% sucrose (n=11) or 10% honey (n=14).*

10.3.2 Y Maze

Testing in the Y maze was carried out every three months and all animals participated in the task at the 3, 6 and 9 month time points. However, due to the need to perform several tasks at the 12 month time point, only 10 rats from each diet group performed the task during this session.

The majority of animals performed the task well, quickly leaving the start arm after being placed in it at the beginning of Phase two (where they were given access to all three arms). However, many animals only explored the arms of the maze for a short period of time before sitting in an arm and not exploring further (this has been reported by other authors also; Conrad *et al.*, 1996). For this reason, the key endpoints for this task were deemed to be 'the arm of first choice' (the arm the animal first chose to enter after leaving the start arm for the first time in Phase 2) and the duration and frequency of visits to each arm during the first 60 seconds. The complete Y maze data for the three diet groups at each of the four testing sessions is given in Appendix 2.

The arm of first choice data (see Table 10.2) demonstrates that honey-fed rats chose to first explore the novel arm rather than the other arm significantly more often during the 3 (χ^2 (1) = 11.3), 6 (χ^2 (1) = 9.3) and 9 (χ^2 (1) = 8.1) month

testing sessions (all $p < 0.001$), with the 12 month data also nearing significance ($p = 0.058$). In contrast, sucrose-fed rats entered the novel arm as the arm of first choice significantly more often than the other arm only at the 3 month testing session (χ^2 (1) = 7.1; $p < 0.01$), with the choice of entry into the novel or other arm of the Y maze approached 50% for the remaining testing sessions. Similarly, rats fed the sugar-free diet demonstrated a clear preference for the novel arm as their arm of first choice during the 3- (χ^2 (1) = 7.1; $p < 0.01$) and 6-month (χ^2 (1) = 4.6; $p < 0.05$) testing sessions only.

A comparison of the percentage of rats choosing to enter the novel arm first at each of the four time points revealed a significant difference between honey- and sucrose fed rats at the 6 month testing session (χ^2 (2) = 8.3; $p < 0.05$; see Table 10.2). At nine months this trend continued (approaching significance) with more honey-fed rats entering the novel arm first compared to either of the other groups (χ^2 (2) = 4.92, $p = 0.08$). However, at 12 months there were no significant differences between the three groups.

Table 10.2: *Arm of first choice (% of animals) after being placed back into the start arm (facing the centre) at the start of Phase 2 where the animal was given access to all three arms.*

	Sugar Free		**Sucrose**		**Honey**	
	Novel	**Other**	**Novel**	**Other**	**Novel**	**other**
3 months[a]	86	14†	86	14 †	93	7‡
6 months[b,e]	79	21*****	57	43	92$^{\tau}$	8‡
9 months[c]	64	36	64	36	87$^{\theta}$	13‡
12 months[d,e]	78	22	60	40	80	20

$^{\tau}$ $p < 0.05$ versus the sucrose-fed rats for the number of rats choosing the novel arm first

$^{\theta}$ $p < 0.10$ versus the sucrose- and sugar free-fed rats for the number of rats choosing the novel arm first

***** $p < 0.05$, † $p < 0.01$, ‡ $p \leq 0.001$ that the observed n < the expected n (the expected n being 50%)

[a] n= 14, 14 and 15 for animals fed the sugar-free, sucrose and honey-based diets

[b] n= 14, 14 and 13 for animals fed the sugar-free, sucrose and honey-based diets.

[c] n= 14, 14 and 15 for animals fed the sugar-free, sucrose and honey-based diets

[d] n= 9, 10 and 10 for animals fed the sugar-free, sucrose and honey-based diets.

[e] One animal from the honey-fed group (6 months) and one animal from the sugar free-fed group (12 months) were excluded from the analysis as they did not enter either the 'other' or 'novel' arm after leaving the start arm.

A repeated-measures analysis of the data from the first 60 seconds of the Y maze task (n = 10 for each diet group) resulted in few statistical differences. There was an overall effect of time, with the duration of time spent in the centre area of the maze being statistically higher at the 6-month testing session compared with the other sessions ($F(2.3,78) = 4.1$; $p < 0.05$). An effect of diet, irrespective of time, was observed only for the frequency of visits to the novel arm, with sucrose-fed rats entering the novel arm significantly more often than those fed the sugar-free diet (1.54 *vs* 1.26 visits; $F(2,26) = 3.9$; $p < 0.05$). Furthermore, an interaction of diet *x* time was observed for the frequency of visits to the novel arm ($F(6,78) = 2.4$; $p < 0.05$), however, further analyses could not clearly differentiate this further, suggesting that this was likely a chance result.

No statistically different effects of diet, time or diet *x* time were observed for the start latency (the time until the animal left the start arm for the first time), the novel latency (the time until the animal first entered the novel arm) nor the frequency of visits or the duration of time spent in the three arms of the maze during any other the other time bins.

10.3.3 Open Field Task

The Open Gield task was performed at 6 and 12 months to determine if there were any effects of diet on locomotor activity. As mentioned in Section 10.2.4.2, the data was analysed for the entire 5 minute (300 second) testing session, as well as for the first and second 150 second intervals. Ten animals per diet group performed the task at 6 months, with 12 animals per diet group performing it at 12 months. However, due to the fact that the two rats that died later in the study participated in the 6 month testing session only, and the fact that different subsets of animals were assessed at the two time points (6 and 12 months), only 8, 9 and 8 animals, respectively, from the sugar-free, sucrose and honey groups participated in both sessions of this task. Full data for the 6-month and 12-month testing sessions (including all animals and time bins) is given in Appendix 2.

A repeated-measures analysis of the entire 300 second data indicated that there was a significant effect of time ($F(8,21) = 5.1$; $p < 0.01$), irrespective of diet, with animals generally exhibiting less locomotor activity at the 12 month session compared with the 6-month session (see Figure 10.9). Overall, the mean number of visits to the middle ($F(1,21) = 9.0$; $p < 0.01$) and outside zones ($F(1,21) = 7.3$; $p < 0.05$) of the arena was significantly higher at the 6-month testing session compared to the 12-month session. In addition, the mean number

of line crosses in the outside zone of the arena decreased by half ($F(1,21) = 32.6$; $p < 0.0001$), and the mean number of total moves in the arena (including zone entries and crosses inside a zone) decreased by 40–50% ($F(1,21) = 41.6$; $p < 0.0001$) during the second 6 months of the study.

Importantly, no significant effect of diet or diet *x* time were observed for any of the parameters measured in the Open Field task (refer to 10.2.4.2 for details) when analysing the data from the entire 300 second testing interval.

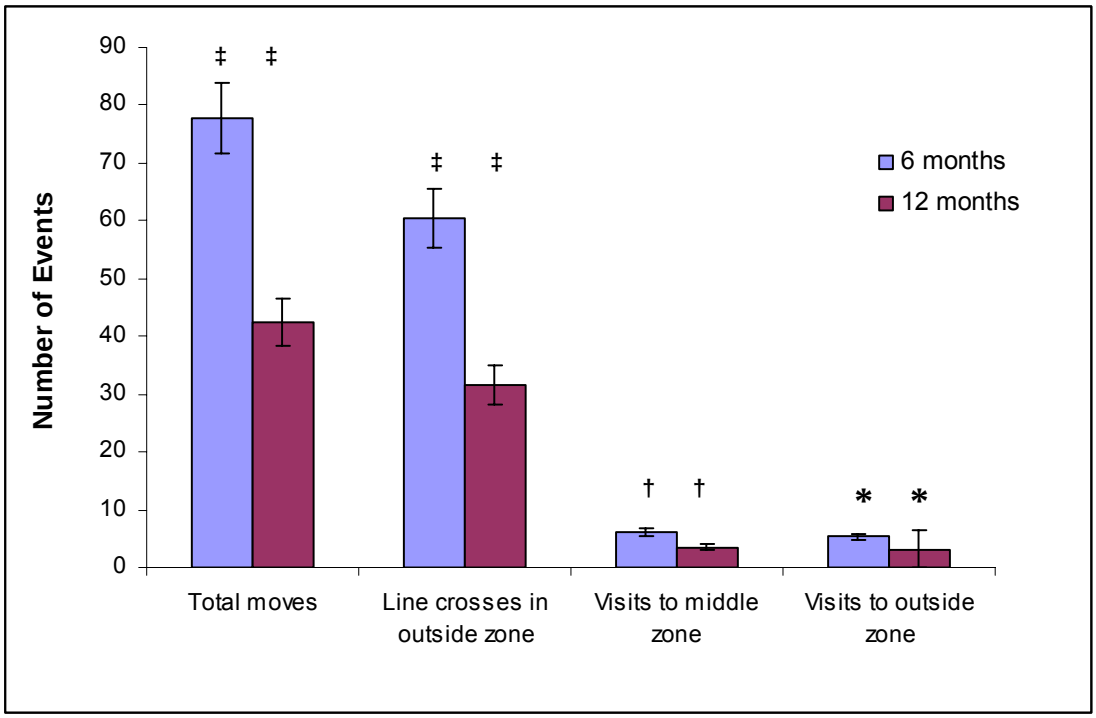

Figure 10.9: *The effects of time observed between the 6-month and 12-month testing sessions of the Open Field task when analysing the data from the entire 300 second testing interval. N = 8, 9 and 8 for sugar-free, sucrose- and honey-fed rats. * $p < 0.05$, † $p < 0.01$, ‡ $p > 0.0001$ between 6- and 12-months.*

An analysis of the 0-150 and 151-300 second testing intervals revealed similar results. Overall, the mean number of total moves was significantly lower during both the first ($F(1,21) = 47.0$; $p < 0.0001$) and second ($F(1,21) = 11.1$; $p < 0.01$) halves of the testing session at 12 months compared with 6 months, and both intervals showed no interaction of diet *x* time.

Further analyses at the individual time points (6 and 12 months) indicated that whereas there were no differences between diet groups at 6 months, statistically significant differences did occur between diet groups in the 12 month

testing session. During the second half of the testing session (151-300 seconds), rats fed the sugar-free diet entered the middle and outside zones of the arena significantly more often than either the sucrose- or honey-fed rats (all $p < 0.05$); however, there was no significant difference in the duration of time spent in each zone (see Figure 10.10).

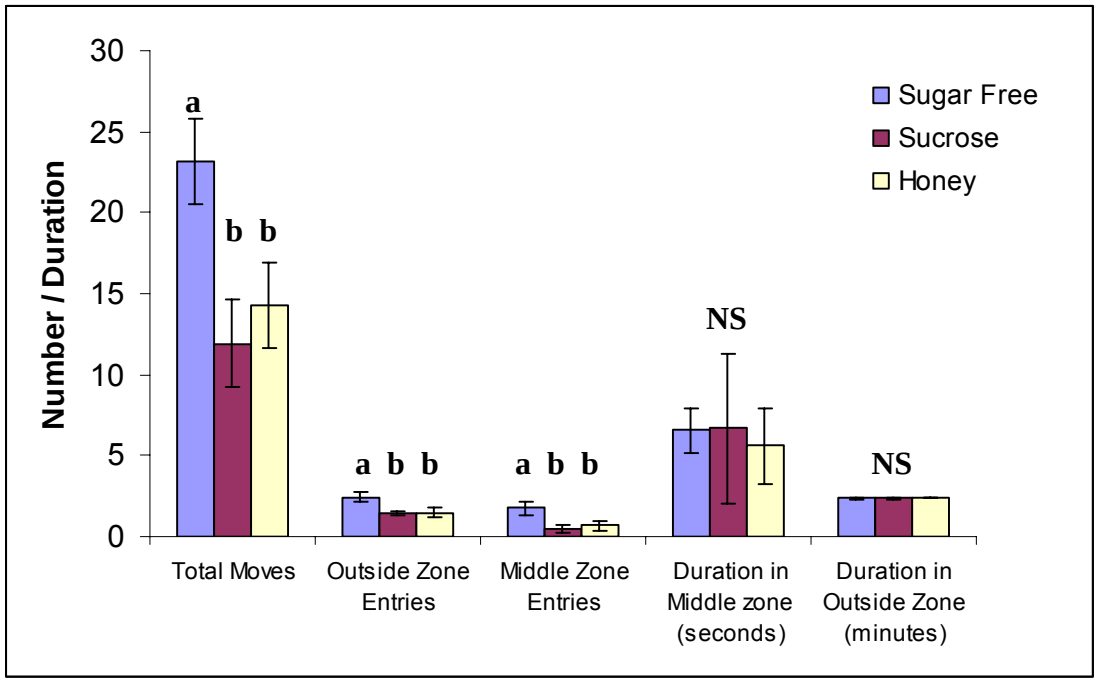

Figure 10.10: *Mean (± SE) 12-month Open Field data during the second half of the testing session (151–300 seconds) for rats fed diets that were either sugar free or contained 8% sucrose or 10% honey (honey is 20% water). Bars not sharing the same letters (within each set of three) are significantly different, p < 0.05; NS = non-significant. 'Total moves' includes all zone entries and line crosses within zones. N = 12 for each diet group.*

Rats fed the sugar-free di*et* also exhibited approximately twice as many total moves (including all zone entries and line crosses within zones) in the arena than sucrose-fed rats (23.2 *vs* 11.9 moves; $F(2,33) = 4.9$; $p < 0.05$). The mean number of total moves was similar in sucrose- and honey-fed rats (11.9 and 14.3 moves) suggesting that both groups may have demonstrated decreased locomotor activity during the second half of the session at 12 months (see Figure 10.10).

10.3.4 Object Recognition Task

Ten animals from each diet group performed the Object Recognition task, following immediately after the Open Field task (the 5 minutes spent in the arena during the Open Field was considered to be the habituation phase (Phase 1) of the Object Recognition task; see Appendix 2 for the raw data). Results showed that during Phase 2 of the task, where the animals were presented with two identical objects, no effects of time or diet were observed for either the number of visits to each object nor the duration of time spent exploring them (See Figure 10.11). Overall all animals in the three diet groups explored objects 1 and 2 to the same extent and there was no difference in the latency to visit the two objects.

During Phase 3 of the task (where one of the original objects was replaced with an identical copy and one was replaced with a novel object) the novel object was visited more frequently ($F(1,22) = 15.8$; $p < 0.001$) and explored for a longer period of time ($F(1,22) = 41.8$; $p < 0.0001$) than object 3 overall, but there were no differences between the three dietary diet groups. Similarly, the latency to visit object 3 and the novel object different significantly overall, the time until the novel object was first explored being approximately a third of that of object 3 (16.4 *vs* 48.4 seconds, $F(1,17) = 12.9$; $p < 0.01$). No interaction of object *x* diet was observed for object latency.

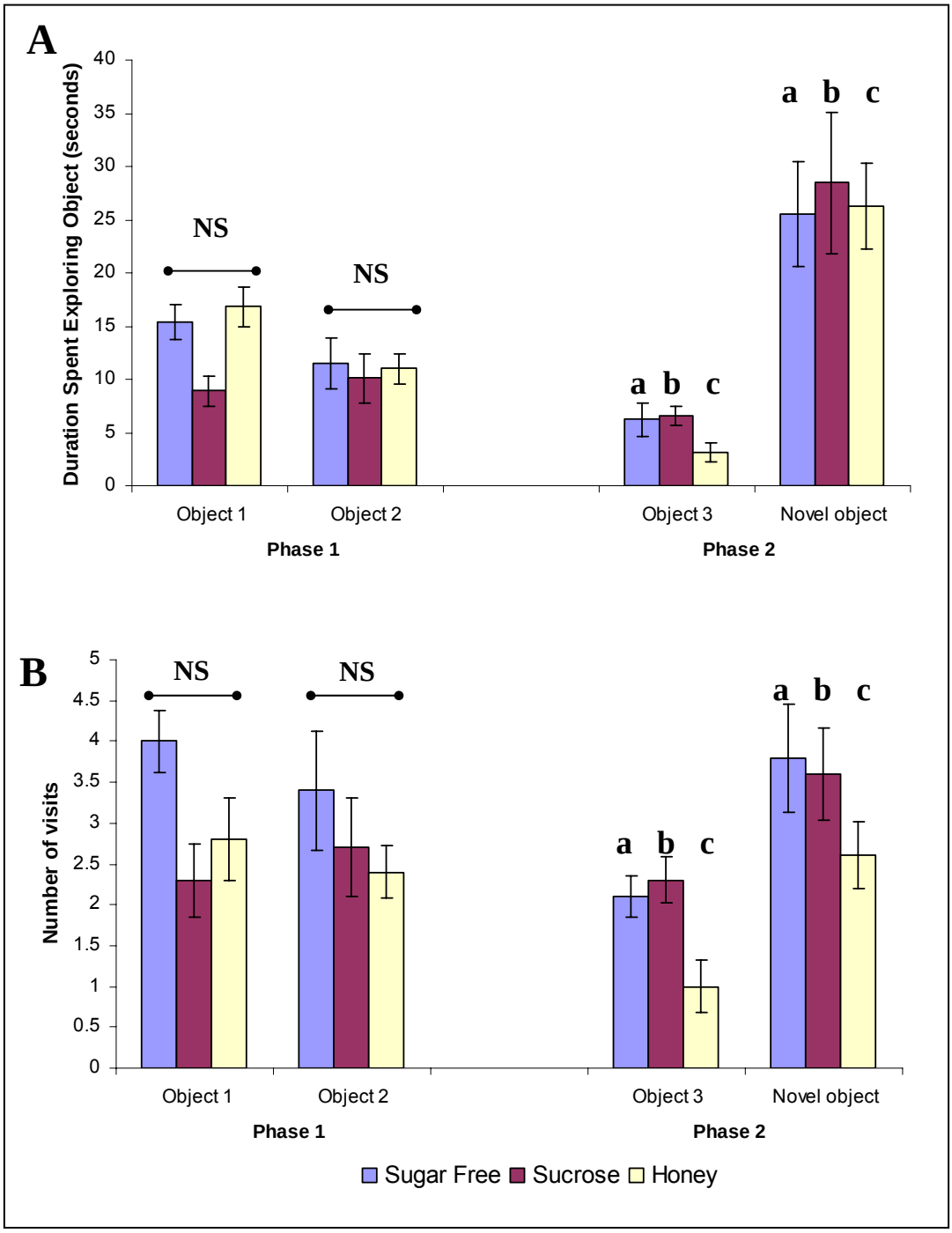

Figure 10.11: *Mean data (± SE) for the Object Recognition task including the duration of time spent exploring each object (A) and the number of visits to each object (B). During Phase 2, animals were allowed to explore two identical objects (object 1 and 2). During Phase 3, the objects were replaced with an identical copy of object 1/2 (object 3) and a novel object. N = 8, 8 and 9 for rats fed a sugar-free, 8% sucrose or 10% honey diet. Bars sharing the same letter are significantly different from each other (all p ≤ 0.001); NS = non-significant.*

10.3.5 Light Extinction Task

Ten animals from each diet group performed the Light Extinction task, and the data was analysed for the complete 20-minute testing session, as well as the first and second 10-minute intervals (see Appendix 2 for the raw data).

The overall number of level presses did not differ significantly between the three diet groups. However, further analysis revealed that, irrespective of diet, the rats pressed the active lever significantly more often than the inactive lever ($F(1,27) = 36.5$, $p < 0.001$) over the entire 20-minute session as well as during the first (0–10 minutes; ($F(1,27) = 13.49$, $p < 0.001$), and second half (10-20 minutes; $F(1,27) = 24.58$, $p < 0.001$) of the testing session. In addition, analysis of each separate diet group confirmed that animals fed on each diet pressed the active lever significantly more than the inactive lever during the entire 20-minute session ($t(9) = 0.01$ (sugar-free), 0.04 (sucrose) and 0.02 (honey) see Figure 10.12).

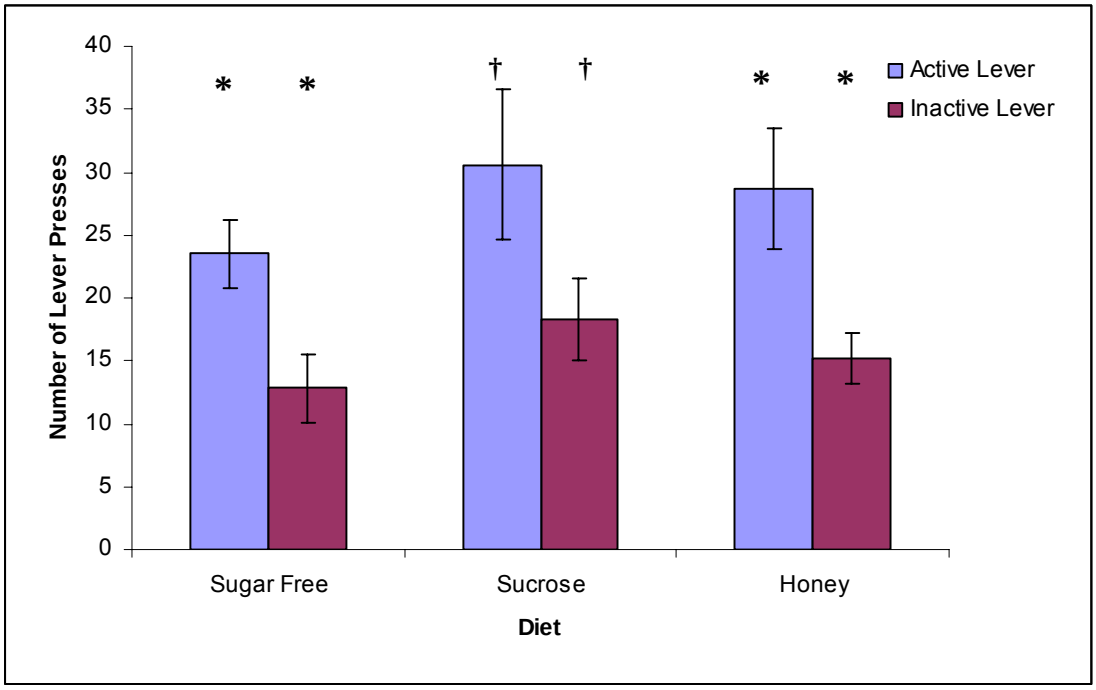

***Figure 10.12**: Mean number (± SE) of total presses of the active lever and inactive lever during the total 20 minute session for rats fed a sugar-free (n=10), 8% sucrose (n=10) or 10% honey diet (n=10). * $p \leq 0.01$, † $p < 0.05$ between the number of active and inactive lever presses.*

Similar results were also obtained for the 0-10 minute session ($t(9) = 0.012$ (sugar-free) and 0.016 (honey)) and 10-20 minute session ($t(9) = 0.003$ (sugar-free), 0.008 (sucrose) and 0.019 (honey) for most treatments. However, the difference between active and inactive lever presses did not reach statistical

significance for sucrose-fed rats during the first 10-minute session. This could indicate that animals fed sucrose were slower to learn that the active lever rather than the inactive lever had to be pressed to turn the lights off, or it could simply be a result of data variability.

The rats in all three diet groups displayed a similar number of active lever presses while the lights were on (AL_{ON}) and while the lights were off (AL_{OFF}) during the entire 20-minute testing session as well as the first and second 10-minute sessions (see Figure 10.13). As a result the AL_{ON} /AL_{TOTAL} ratios (proportion of the total active lever presses that occurred while the lights were on) were close to 0.5 for all groups, indicating that the distribution of active lever presses between the lights on and the lights off periods was approximately even (a series of one-way between-groups ANOVAs confirmed that there were no significant differences between the groups during either test session). This suggests that whereas the rats were able to determine that pressing the active rather than the inactive lever turned off the lights, none of the three dietary groups were able to distinguish the difference between pressing the active lever while the lights were on and when the lights were off (*i.e.* that pressing the lever whilst the light were already off had no further effect).

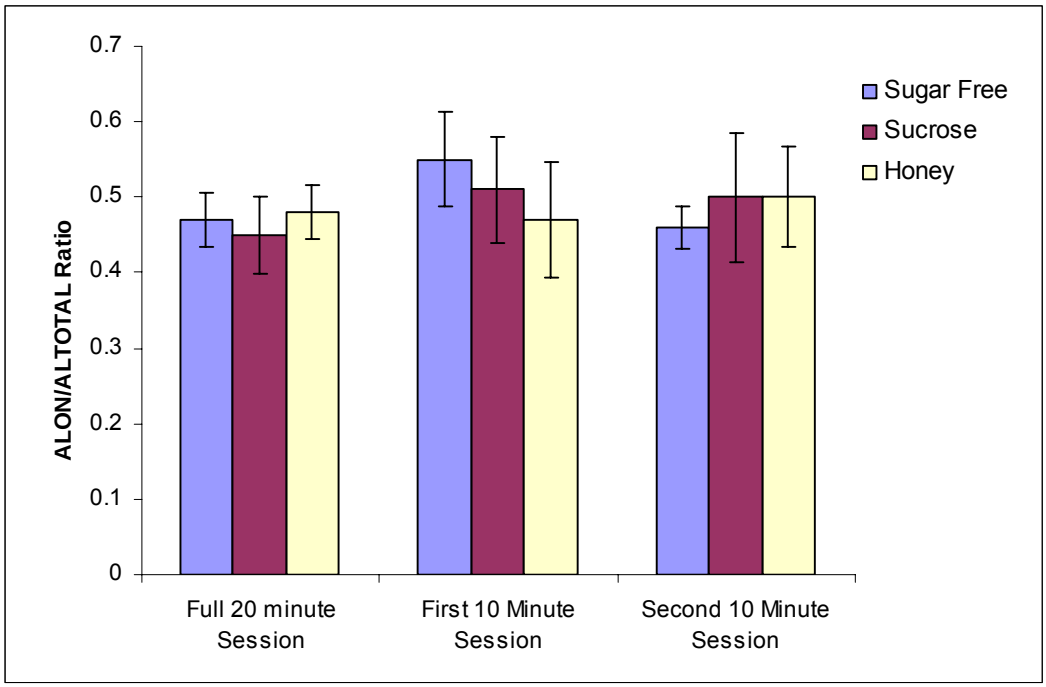

Figure 10.13: *Mean (± SE) number of active lever presses while the lights were ON (AL_{ON}) as a proportion of the total number of active lever presses (AL_{ON} / AL_{TOTAL} ratio) during the total 20-minute session, and the first and second 10-minute sessions for rats fed a sugar-free, 8% sucrose or 10% honey die (n = 10 per diet group).*

10.4 DISCUSSION

It appears that consuming honey rather than sucrose as a part of the daily diet may have an effect on various aspects of cognition as well as on other age-related disorders (described in Chapter 9). In particular, honey-fed rats outperformed their sucrose- and sugar-free-fed counterparts in the EPM, spending approximately twice as long in the open arms of the maze at each time interval tested (3, 6 and 9 months). The cause of anxiety is a complex issue, although it occurs in both animals and humans (Stone, 1997). Research has suggested that several areas of the brain may play a role in the development of anxiety (Bridges, 2006), although the full pathology has yet to be elucidated. The ventral hippocampus of the brain, for example, has been shown to possibly be involved as lesions in that area result in a reduction in anxiety behaviour (Bannerman *et al.*, 2004). As well several other areas of the brain have been implicated in the etiology of anxiety, including the locus coeruleus, amygdala, hypothalamus and higher cortical areas (Charney *et al.*, 1998; Gray and McNaughton, 2003). Importantly, however, it has been suggested that generalised anxiety disorders may result from a disruption to the neurotransmitter systems as agents acting at these sites (*e.g.* Benzodiazepines) decrease the anxiety response seen in both animals and humans (Charney *et al.*, 1983; Teixeira-Silva *et al.*, 2004). Although not proven scientifically in recent years, one study from the 1950s (Goldschmidt and Burkert, 1955) has suggested that honey may contain compounds that exhibit hormonal or neurotransmitter activities. These authors reported that honey acts as if it contains acetylcholine and choline esters (which would bind to muscarinic and nicotinic receptors). Benzodiazepines, in contrast, bind to γ-aminobutyric acid (GABA) receptors suggesting that different molecules would be involved. It is possible, though, that honey may also contain compounds that could bind to GABA receptors, and in light of the findings in the current study it would certainly warrant further investigation. Lyons and Truswell (1988), for example, have reported that high carbohydrate meals can influence serotonin synthesis. Thus, it is not infeasible to expect different carbohydrates to also influence other brain receptors.

Of course, honey could also be affecting anxiety in ways that do not involve neurotransmitter activities. It is possible that honey may have reduced anxiety-like behaviour because of its high antioxidant content. Evidence suggests that oxidative damage is critically involved in the pathological manifestations of aging including cognitive performance (Kumar and Gupta, 2002), however, a review of the literature suggests that only a few studies have investigated what

effect antioxidants may have on anxiety. In a recent study (Bouayed *et al.*, 2007a) it has been demonstrated that clorogenic acid, an antioxidant compound present in fruits, decreased anxiety-related behaviour in mice using an EPM. Similarly, *Vaccinium* berries (with a high antioxidant content) have been shown to reduce anxiety-like behaviour in mice (Barros *et al.*, 2006), and Whortleberry extract (Kolosova *et al.*, 2006b) and Zingicomb (a mixture of Zingiber officinale and Ginko Biloba extracts; Hasenhohr *et al.*, 1996) have reduced anxiety scores in rats. Furthermore, recent research has suggested that there may be a definitive link between oxidative stress and levels of anxiety, as anxiety-related behaviour was higher in mice with elevated levels of reactive oxygen species (Bouayed *et al.*, 2007b). High levels of anxiety appeared to also be correlated with an increase in free radical intake and a decrease in plasma antioxidant content in one clinical study involving 66 women (Arranz *et al.*, 2007). Importantly, though, one study has shown that the behavioural response to antioxidant content may differ depending on the strain of animal used (Kolosova *et al.*, 2006b). In that study, Whortleberry extract reduced EPM-based anxiety in OXYS rats after 45 days of feeding but *increased* anxiety behaviour scores in Wistar rats given the same extract during the same period. Thus, it appears that whilst the antioxidant content of honey may have contributed to the reduced anxiety scores in the animals in the present study, its significance requires further evaluation before definitive conclusions can be drawn.

Research has shown that when placed on an EPM, a rat's behaviour is largely based on its anxiety level. Normal rats that have not been given anti-anxiety treatments become moderately anxious in the new environment, preferring the closed arms over the less secure open arms (Salum *et al.*, 2000). In contrast, animals given anti-anxiety drugs tend to be less anxious, spending more time in the open arms of the maze (Treit *et al.*, 1993), with generally less movement overall (Kalynchuk and Beck 1992). It is also possible, therefore, that honey maybe having an anxiolytic or pharmacological (tranquilising) effect, rather than preventing a decline in cognitive function, and that this may account for why the honey-fed animals were substantially less anxious at each time point compared with rats fed the other two diets.

The ability of honey to decrease anxiety levels is not always desirable though as anxiety is a response that is designed to enable an individual to deal with threatening situations and perform at optimum levels (Bridges, 2006). That said, however, there are many anxiety disorders that present worldwide, where an abnormal anxiety response is generated to normal everyday situations, the

level of anxiety being out of proportion to the level of stimulus generating the response (Bridges, 2006). Indeed, it has been reported that anxiety disorders now account for 27% of all psychiatric illnesses, with a cost of over 41 million Euros per annum in Europe alone (Andlin-Sobocki *et al.*, 2005). Several diet groups are available for individuals suffering from such disorders, although today the mainstay therapy is still the use of anxiolytic drugs (Bridges, 2006). However, such drugs are often associated with unwanted side effects, and in some patients (particularly those with mild anxiety), their sustained use is not warranted. The ability to influence anxiety levels through natural supplements and diet is therefore a valuable tool for patients wishing to control their anxiety.

In addition to the effects of diet on EPM activity observed in this study, it was also shown that there was a significant effect of time overall, with animals generally spending more time in the open arms as they got older. It is possible that there was an overall increase in the time spent in the open arms of the EPM as the maze was no longer unique after the first testing session. An analysis of the literature shows that EPM tasks are generally only performed once (Rodgers and Johnson, 1998; Cole and Rodgers, 1995; Sayin *et al.*, 2004), and not at reoccurring time points as in this study, thus there is little evidence to either support or reject this theory. However, it is well established that the memory of a rat is in the range of hours to days rather than months (Conrad *et al.*, 1996; Levin *et al.*, 1996), thus it is unlikely that the animals remembered the task three months after a previous session. More likely, these results were accurate, indicating that increasing age leads to a decrease in anxiety levels. Indeed, these findings agree with those from other animal studies (Slawecki, 2005; Chen *et al.*, 2007) as well as with some clinical studies (Le Roux *et al.*, 2005; Brenes, 2006) that show that anxiety decreases with age.

Interestingly, a significant finding was also observed in the Y maze task, with honey-fed rats choosing to enter the novel arm more often than the other arm for 9 months compared with only 3 and 6 months for rats fed sucrose and a sugar-free diet, respectively. This suggests that the honey-fed rats did not show the reduction in spatial memory that was exhibited by rats fed the other two diets, though it must be noted that a significant difference between diet groups was only observed at 6 and 9 months (refer to Table 10.2). Rats fed honey appeared to recognise the novel arm as being the unvisited arm of the maze at each of the four testing sessions (although the difference between novel (80%) and other (20%) arm only neared significance at 12 months) whereas rats fed sucrose only recognised the novel arm as being the unvisited arm at the 3-month testing

session. For the remaining three testing sessions, the ratio of sucrose-fed animals entering the novel and other arms of the maze neared 50:50. With animals fed the sugar-free diet, the novel arm was recognised as being the unvisited arm at the 3- and 6-month testing sessions. This would suggest that GI may have influenced recognition memory in this study as both the sugar-free- and honey-based diets were low GI, whilst the sucrose-based diet was high GI. Furthermore, a look at the data at 12 months shows that the percentage of animals fed the sugar-free diet entering the novel arm (78%) was very similar to the percentage of honey-fed rats (80%) entering the novel arm at that time. Although the difference between the proportion of animals entering the novel and other arms did not approach significance at 12 months for rats fed the sugar-free diet ($p = 0.096$), it is possible that any significant difference between diet groups was masked by the reduced number of animals tested at that time (only 9, 10 and 10 rats from each diet group completed the task at this time point). Thus, it could be that rats fed honey or the sugar-free diet had a similar level of recognition memory after 12 months of feeding, with both groups outperforming the animals fed the sucrose-based diet.

HbA1c levels were also significantly reduced in honey-fed rats compared with those given sucrose at the end of the study (refer to Section 9.3.2), lending weight to the theory that rats fed honey may have demonstrated better cognitive abilities at 12 months because of improved glycemic control. As discussed in Section 10.1, impaired glucose tolerance and hyperglycemia have been shown to have an impact on cognitive function, with diabetic individuals and those suffering with poor glycemic control often exhibiting a greater impairment of cognitive function in older age (Messier *et al.*, 2005; Di Bonito *et al.*, 2007). In addition, Awad *et al.* (2004) specifically reported that impaired glucose tolerance has a significant impact on various aspects of memory, and animal studies have shown that regulation of insulin and glucose levels improves memory (Stone *et al.*, 1990) and maze performance (Long *et al.*, 1992) in aged rats. It is therefore possible that the lower GI of honey prevented the decline in spatial memory seen in rats fed sucrose.

However, the HbA1c levels of rats fed the sugar-free diet, whilst numerically lower, were not statistically different ($p = 0.249$) from those of sucrose-fed rats at the completion of the study. This suggests that glycemic control was not completely responsible for why honey-fed rats exhibited better spatial memory for longer periods of time (as both the sugar-free and honey diets

should be low GI compared with sucrose). Thus, other components of honey may also play a role in the improved memory scores seen in this task.

As with most aspects of brain function memory is a complex process, although it is well established that it can be influenced by diet (Greenwood and Winocur, 1990; Yokogoshi and Nomura, 1991; Long *et al.*, 1992; DeSilvey, 2005; Booij *et al.*, 2006). Many studies have reported a significant effect of dietary antioxidants on cognitive performance (see Section 10.1), and a number of studies have even shown a direct correlation between antioxidant intake and improved memory scores (Perkins *et al.*, 1999; Raghavendra and Kulkarni, 2001; Cho *et al.*, 2003; Farr *et al.*, 2003). In light of these studies, it is possible that the antioxidant content of the honey may be, in part, responsible for the improved spatial memory seen in honey-fed rats in the Y maze task. The honey used in the present study was specifically chosen for its high antioxidant content (TEAC = 3.1 mmol/l), and this level of antioxidants is comparable to that in the diet in another study that has shown a significant effect on cognitive function (Cho *et al.*, 2003). However, the majority of studies investigating the effect of antioxidants on cognition do not report the antioxidant content of the foods/extracts they are investigating but instead give information as grams of food extract (Bickford *et al.*, 2000; Guilherme dos Santos *et al.*, 2005; Kolosova *et al.*, 2006a). Thus it is not possible to fully compare the effects of honey versus other dietary substances on cognitive performance.

Interestingly, there were generally few significant differences between diet groups for the amount of time spent in the three arms of the Y maze, nor in the number of visits to each arm. It had been hypothesised at the start of this trial that if spatial /recognition memory were to differ between diet groups, that there would be a noticeable difference in the amount of time spent in the novel arm of the maze, particularly in the first 1–2 minutes. However, with the exception of the frequency of visits to the novel arm at the 6 month testing session, no differences were observed for either the number of visits to the novel arm, or the duration of time spent exploring that arm of the maze. An inter-trial period of four hours was chosen for this task, as it has previously been reported that Sprague Dawley rats demonstrate no recognition memory after six hours (Taghzouti *et al.*, 1999) but that recognition memory occurs for time periods of four hours or less (Conrad *et al.*, 1996). It is possible that four hours was right at the limit of the animals ability to remember the maze and the spatial cues, and that a shorter inter-trial period may have improved the performance of some or all animals in this task. It was not possible to investigate other inter-trial intervals during this study due to the

time it took to complete this task at each testing session, nor could pilot studies been effectively undertaken as it was unknown how the performance of the animals would change as they aged. Based on these results, however, it would be advantageous to maybe use a shorter inter-trial interval if similar studies were to be carried out in the future.

Another compounding factor with the Y maze task is that the majority of animals did not explore the Y maze to any great extent (exploration was deemed to include movement around the maze/arm entries as well as sniffing), most rats instead preferring to sit in an arm for a prolonged period of time. It is likely that this occurred as the maze (and the novel arm) quickly became a familiar environment with no food or other rewards to entice them into exploring further. Other studies have demonstrated that exploration in maze tasks may be improved by the presence of food rewards (Seo *et al.*, 1999; Sayin *et al.*, 2004) though as mentioned earlier it was not possible to use food rewards in any of the behavioural tasks in the present study due to the need to accurately control and record dietary intake and weight gain. In addition, the maze had relatively narrow and high-sided arms, providing the animals with a level of security. This may have provided an environment where the animals did not feel the need to explore the maze any further than was required to determine the presence of food, predators or a means of escape.

In contrast, when the animals were placed into the large open area of the Open Field arena the rats generally moved around constantly during the 5-minute session (albeit slowing over the time of the task), most likely in an attempt to find somewhere to hide. However, there was no effect of diet on the degree of locomotor activity during this task at either 6 or 12 months. This indicates that the degree of movement displayed by animals in the various behavioural tasks in this study was likely to not have been influenced by diet, but rather was due to the maze designs themselves. Thus, any differences observed in the Y maze, EPM or Object Recognition tasks could be assumed to be real findings and not a result of the fact that one dietary diet group exhibited a higher lever of activity/mobility compared with the other diet groups.

As expected, the degree of movement within the Open Field arena decreased as the animals aged, with the older animals exhibiting fewer movements within the arena (see Figure 10.9). This agrees with the observations that older individuals tend to be less active compared with their younger counterparts, this being affected by factors including weight gain, changes in

lifestyle/fitness and general age-related muscle, tissue and bone degeneration (Hayflick, 1998).

No differences between diet groups were observed for animals performing the Object Recognition task, although all animals recognised the novel object as being the new, unvisited object. These results suggest that the honey diet had no impact on the degree of object recognition, although it may be that the animals were not old enough that either the control sugar-free-fed animals or those given sucrose displayed a decline in recognition memory. At what age a rat is considered to be "old" is debatable, although Hubert *et al.* (2000), have reported that Sprague Dawley rats fed *ad libitum* will often live for 2–3 years in a laboratory setting, with death generally occurring from approximately 50 weeks of age onwards. Similarly, Altun *et al.* (2007) have reported that the mean survival age of Sprague-Dawley, inbred female Lewis and outbred male Wistar rats is 29–30 months. In a number of studies assessing behavioural measures in rats the animals were considered to be "old" when aged 22–25 months (McLay *et al.*, 1999; Topic *et al.*, 2002; Bizon and Gallagher, 2003). In addition, one paper has stated that the rats were "young" when aged approximately 7 months and "middle-aged" when aged 10 months (Bizon and Gallagher, 2003). In the current study animals were aged approximately 14 months at the completion of the study, which would put them at middle–old age, and this should have been old enough for declines in recognition memory to have begun to occur (and indeed a decline in recognition memory was observed in the Y maze task). Thus it is unknown why rats that completed the Object Recognition task did not show a loss in cognitive performance, although it may have been that the task was simply not sensitive enough to pick up the minor changes that may have occurred through dietary influence. Alternatively, the power of the study may have been inadequate to detect statistically significant differences. As this task was performed only once at the 12 month testing session, variability within groups was likely to be high due to differences in aging, weight, metabolism *etc (*indeed, the coefficients of variation (COVs) were 35–60% for most variables, and 90% in one case). Thus, it is possible that a greater number of experimental animals may have yielded better results. In addition, object recognition works on the theory that an animal will want to explore a novel object in its environment. It could be, however, that the animals in this study were habituated to the presence of novel objects as enrichment devices were placed in their cages at regular intervals throughout the 12-month study.

Importantly, however, two studies have shown dietary antioxidants to have an effect on object recognition performance (Goyarzu *et al.*, 2004; Andres-Lacueva *et al.*, 2005). In the first study, aged Fischer rats (exact age unknown, abstract only) fed a 2% blueberry-supplemented diet for 4 months demonstrated significantly improved object recognition capabilities compared with aged animals fed a control diet, with blueberry-fed animals having similar levels of cognitive performance to those of young rats fed the control diet. These authors reported that this was due to reduced levels of the oxidative stress responsive protein nuclear factor kappa-B in the brain (Goyarzu *et al.*, 2004). In the second study, Andres-Lacueva *et al.* (2005) reported that 19 month old Fischer rats fed 2% blueberry extracts for 8–10 weeks demonstrated improved recognition and memory compared with control animals, with significant levels of anthocyanins being detected in the brain. Thus, both of these studies have shown that dietary antioxidants are indeed capable of crossing the blood brain barrier, where they may exert positive effects on oxidant-related declines in cognitive performance. It is likely, therefore, that the antioxidants present in honey may also reach the brain where they too can offset oxidative damage. It would be of interest to assess this in future studies.

Although the Light Extinction task has produced good results in other studies (Messaoudi *et al.*, 1996; Benton *et al.*, 2003), it revealed no conclusive findings in the present study. Rats in all three diet group groups appeared to be able to distinguish that the active lever turned the light off whilst the inactive lever did not (see Figure 10.12), although there were no differences between treatments (this may have been due to the small number of rats tested and the high intra-group variability as COVs were in the range of 39–97%). Measurement of learning capability generally involves tasks where there is a strong positive or negative association with a given action (*i.e.* animals are given a mild electric shock when they perform an incorrect action; Torres *et al.*, 2005; Schneider *et al.*, 2006) or where the animals are given training for a particular task (reviewed by Whishaw, 1999). As the ability to learn appears to deteriorate in older age (Cerella *et al.*, 2006; Seidler, 2006; Bennett *et al.*, 2007), it may be advantageous to use more operant-learning based tasks in future studies, or tasks where there is a stronger incentive for the animal to learn. Again, the age of the animals may also have had an impact on the results of this task, and it may be worthwhile in future investigations to assess learning skills in animals aged older than 14 months. However, there are obviously additional costs associated with feeding

and housing animals for an additional 6–12 months and this may be beyond the scope of further studies.

In conclusion, it appears that long-term feeding of honey, sucrose and a sugar-free diet may have differential effects on cognitive performance in rats, with honey-fed rats exhibiting improved cognitive performance in some tasks at the completion of the study. Interestingly, where a difference in cognitive ability was observed between diet groups, rats fed the sugar-free diet generally performed less well than the honey-fed rats. This suggests that the improved performance seen in honey-fed rats is not due only to a lower dietary GI content (and better glycemic control) but involves other components in the honey. As mentioned previously, the honey chosen for this study contained a high content of antioxidants, and it is likely that these were involved in the improved cognitive abilities seen in honey-fed rats, as other studies have demonstrated the efficacy of antioxidants on cognitive performance *in vivo*. It would be of interest to assess honeys of differing floral sources and those with differing antioxidant contents to ascertain what other factors present in the honey may be responsible for the results obtained in this study. Importantly, though, this study has demonstrated that the cognitive decline often observed in older individuals may be able to be offset by long-term improvements to diet in earlier life.

Chapter Eleven:

General Discussion

During the research presented in this thesis a number of interesting results have been obtained. However, like all scientific studies/trials, those presented in this thesis are also open to criticism and evaluation. This chapter will therefore critically review the findings of this thesis, specifically the use of animal models and how the results of these studies may relate to human health.

11.1 USE OF ANIMAL MODELS FOR NUTRITIONAL INVESTIGATIONS

Animal models have been used extensively in this thesis to investigate the various endpoints, and several conclusions have been drawn based on the data obtained. However, whereas animal models offer advantages in nutritional investigations (see below) they also can have negative implications. In particular, whilst some animals share certain characteristics and conditions with humans, they are only ever analogues at best and not an exact match. Differences in anatomy, physiology and pathology can all contribute to why results may differ in a given animal model compared with a human counterpart. LaFolette and Shanks (1992) have reported that animals are generally chosen for a given study if they are shown to be a “strong” model, *i.e.* if they are identical to the analogous human features in all casually relevant aspects, such that the research generated

from such models can be confidently applied to humans. But, investigations into disease and physiological disorders often do not meet these criteria. Cancer, for example occurs in both animals and humans, and initial thoughts would suggest that a rodent or primate model could effectively be used to investigate carcinoma formation. However, development of cancerous malignancies can differ widely depending on their causes (Kaufman *et al*., 1989), and experimentally-induced malignancies in non-human animals cannot be directly compared to those that occur spontaneously in humans.

In this thesis, the basis of most of the studies has been to investigate possible differences in sugar absorption and metabolism (*i.e.* the fructose and glucose in honey compared with sucrose and amylose) and to determine whether other components of honey (particularly antioxidants) may be absorbed from the gut to confer a physiological effect. Whilst rats are generally considered to be good models for humans, particularly with respect to nutrition, they are not always a perfect match. Recent research (published after much of the work in this thesis had been completed) has reported, for example, that the internal structures of insulin-producing pancreatic islet cells are dramatically different between rodents and humans (Cabrera *et al*., 2006), and that fructose transport and metabolism in adipose tissue is compromised in some rat models compared with humans (Litherland *et al*., 2004). Furthermore, several studies have demonstrated that feeding a high fat diet (as occurred in this study) to some breeds of rats (Sprague Dawleys were not investigated) can lead to insulin resistance and impaired glucose metabolism (Kraegen *et al*., 1991; Bernstein *et al*., 1977; Furler *et al*., 1997; Han *et al*., 1997). In addition, rodents are generally considered to not be an appropriate model to assess lipoprotein concentrations in blood (this was a key endpoint in several studies) as plasma lipid levels in rats are only minimally affected by modifications of dietary fat and cholesterol compared with their response in humans (Hegsted, 1975). However, more appropriate models for both blood sugar- and lipid-related endpoints would likely be animals with a similar and comparable physiology (pigs and primates), and the burdening costs associated with the housing, feeding and manipulation of these animals would make many of these studies impractical and unachievable.

Despite the differences that can occur, the use of small animals in biomedical research is still warranted as it provides a means of undertaking research that is cost- and time-effective, allowing the generation of huge amounts of data that could not be obtained otherwise. It should be noted, though, that such studies are generally considered to be pilot investigations and most

researchers do not automatically draw direct conclusions as to the implications their findings could have in humans. Instead, such animal studies provide an opportunity to investigate a hypothesis or evaluate a new drug in a controlled manner, and further studies are undertaken (including clinical evaluations) once the additional costs, time and interventions are deemed appropriate.

Despite the fact that the use of animal models can be flawed, it has offered several advantages in this thesis. The one main advantage of using rodents in these studies has been the ability to completely control the experimental conditions and diet, thereby providing the best possible means of determining whether there were actual differences between the dietary treatments. To perform such controlled studies in humans would be virtually impossible as it is impractical to expect volunteers to conform to such a rigid dietary regime for extended periods of time. The second main advantage of using rodents was due to the costs associated with this work. Clinical studies are prohibitively expensive for all but the more wealthy companies due to the large number of patients that must be recruited in order to show a significant effect (and because of the large number of withdrawals that usually occur due to side effects) (Mawson, 2003), thus it would have been completely unfeasible to have undertaken the work in this thesis using human volunteers. Thirdly, without the use of small animals it would have been impossible to investigate the efficacy of honey in older age after long-term feeding, as humans (and closely related animals such as primates), have a life span that excessively exceeds that of a PhD project (or even most funded research programs).

As mentioned, the animal trials in this thesis were designed to obtain the best possible data which might indicate whether honey could have an effect on the various endpoints studied (calcium absorption, weight gain, cognitive performance *etc*). It is recognised, though, that irrespective of the fact that animals were used rather than humans, the experimental designs were not real life situations. The calcium studies (Chapters 7 and 8), for example, were designed using male animals only to avoid the impact that hormones might have on calcium uptake, despite the fact that it is primarily women who suffer from osteoporosis and other low-calcium related disorders. As well, in all studies the animals were fairly restricted in the amount of activity they could undertake because of the size of the cages they were housed in, and it is unknown what impact this may have had on the results. It would therefore be advantageous in future work with animal models to more closely match the experimental

conditions to typical human situations so as to better assess what impact honey may have on human health.

11.2 IMPLICATIONS FOR HUMAN HEALTH

As the data from this thesis has demonstrated, honey may have the ability to influence certain aspects of human health. Certainly, there appears to be a consistent finding across many of the studies that ingesting honey may improve an individual's ability to control their blood sugar levels and weight gain. It is possible that this could be due to differences in the glycemic responses that result after eating honey and sucrose, as honey-fed rats often exhibited weight gain and blood glucose levels that were similar to those fed a sugar-free diet that contained low GI amylose. However, it must be noted that the GI value was never actually measured for any of the honey samples used. The GI value of a particular food is generally determined by measuring the area under a two hour blood glucose response curve (AUC) in at at least 10 individuals (measured using 15 minute blood sampling for the first hour, and 30 minute sampling for the second hour) following the ingestion of a fixed portion of the food (usually containing 50 g carbohydrate) (Brouns *et al*., 2005). The AUC value is then divided by the AUC value of the standard (either glucose or white bread) and multiplied by 100. This process is relatively costly, though, and was not performed in this thesis because of budgetary constraints and because of the fact that the literature generally reports that honey has a low to moderate GI value (Shambaugh *et al.*, 1990; Arcot and Brandmiller, 2005; Ischayek and Kern, 2006) and results in low plasma glucose levels after consumption (Samanta *et al.*, 1985; Akhtar and Khan, 1989; Al-Waili, 2003a, 2003b 2004). It would be worthwhile, though, to accurately assess the GI value of any honeys used in further studies so that more accurate conclusions are able to be drawn. As well it would be of interest assess honeys of differing floral sources as limited research has demonstrated that floral source may be a contributing factor to the GI value of the honey (Foster-Powell *et al*., 2002).

Another important factor to consider is that whereas the GI values of honey and sucrose have been shown to differ when assessed as stand-alone dietary ingredients (Shambaugh *et al.*, 1990), it is unknown whether any differences in GI would be detected when they are incorporated into the diets at levels usually used in this thesis (8–10%). For example, the addition of high GI sugars to low GI yoghurts at a level of 15 g /100 g still resulted in a low GI overall

when tested using the standard method (Joanne Todd, Fonterra Brands, personal communication). With the composition of the diets in this study consisting of 36–53% high GI starch, 16–22% milk powder/casein/lactalbumin and 15–16% fat, it is likely that the overall GI of the diets was moderate to high, although without full GI testing, this is hypothetical only. However, assuming that this is correct, the question is then whether adding 10% honey (containing 8% sugars) can actually have an impact on this rating. It may be that honey is not directly influencing weight gain and blood glucose levels through differences in GI, but rather through other mechanisms, including its possible insulin-mimetic effects (see Section 6.4 for details).

A second issue that must be addressed is the fact that in the studies presented in this thesis *all* of the sugars in the diet were replaced with honey. As mentioned previously, the carbohydrate content of the diets in Chapters 6, 8 and 9/10 was based upon data from the 1997 National Nutrition Survey (New Zealand Ministry of Health, 1999) that reported 45–47% of the energy content of the average New Zealander's diet comes from carbohydrate. However, the data was not available as to what this carbohydrate content generally consisted of, *i.e.* whether it was complex carbohydrates such as pasta and whole grains, or more simple carbohydrates such as white flours and sugars. What was reported, though, was the fact that the daily median intake of sugars was 131 g for men and 99 g for women, and that the daily median energy intake was 11 631 kJ and 7 701 kJ, respectively. Assuming an energy content of the sugars of 17 kJ/g, sugars therefore accounted for approximately 20% of the daily energy intake (19% for men and 22% for women). In the present studies the total levels of honey or sucrose added to the diet was approximately 8% by weight (honey was 10%, but 20% of this is water). Coincidentally, this was also equivalent to 8% of the daily energy intake (see Section 9.2.2 for an example of how the dietary energy intake was comprised), indicating that the quantity of sugar used in the present studies was only about half of that of the typical New Zealand daily intake. Importantly, though, the survey data also did not indicate what types of sugars make up the 20% daily kilojoule intake. Differences in the quantities of fructose, sucrose and glucose ingested can, and do, have very different effects on blood glucose levels (Foster-Powell *et al.*, 2002), and the impact that a given persons sugar intake can have on their health can vary greatly, even when not considering the differences in metabolism and lifestyle.

As Fonterra was funding the majority of the research presented in this thesis, the underlying objective was always to assess how honey might act when

it was incorporated into milk-based products such as yoghurts and flavoured milk drinks. A review of the milk products currently available on the New Zealand market revealed that such products generally have around 7.5–9.5% sugars added, these making up the bulk of the carbohydrate content. Thus, in the studies presented in this thesis, a level of around 10% honey (containing 7.9–8.0% sugars) was generally used as it was considered to be the maximum level that would potentially be added to a milk-based product. The problem arises, though, as there is a big difference between 10% honey/8% sugars in a drink or yoghurt consumed once or twice a day and 10% honey/8% sugars in the total diet. However, as mentioned, in the work in this thesis all of the sucrose in the diet was replaced with honey to directly compare what effects the two diets would have on the various endpoints. To have incorporated sucrose into the diet at a level of 20% of the energy intake would certainly have been feasible, but to have that much honey in the diet would have been impractical. Honey is often used as a sugar substitute by those who are health conscious but rarely, if ever, is it used to replace all the sucrose that is being consumed. It may have been advantageous to have used 20% sucrose in one diet and a mixture of honey and other sugars in the other (with the total sugar content equally 20%) but it then would have been impossible to know which effects were due to the honey alone.

Furthermore, the levels of honey consumed by rats in these studies are substantially more than that consumed by the average public. Data from the Global Environmental Monitoring System/ Food Contamination Monitoring and Assessment (FEM/Food) Program indicates that daily consumption of honey per capita ranges from 0.0–1.3 grams per person (World Health Organisation, 1998). This is an incredibly small amount when you consider that it comprises only about 0.2–0.3% of the days total energy intake, suggesting that any positive, health-promoting effects are almost certain not to occur at these levels.

In addition, in all the studies presented in this thesis, raw honey was used, and it is unknown whether the bioactivities observed would be retained after food processing. The addition of honey to milk based products, for example, might require it to undergo pasteurisation, and it is likely that the high temperatures involved (usually 71^{o}C for 15-20 seconds) would alter enzyme structure/function as well as that of other unidentified heat-sensitive molecules. In addition, honeys produced for sale in North America and Europe are regularly pasteurised before being made available, and the consumer must be aware that the effects observed in the various studies in this thesis may not have occurred had we used pasteurised honey. ‘Raw’ honey can be sourced directly from the apiarists as well

as from health food stores, though, and this is a better alternative for individuals wishing to consume honey because of its possible effects on health. In addition is has also be reported that certain honeys can actually retain some of their heat-sensitive properties after heating due to the presence of iron-binding antioxidants (P. Molan – personal communication). Honey Dew and Rewarewa honeys, for example, have been shown to retain their glucose oxidase activity after heating if high levels of these particular antioxidants are present. This work is very much in its infancy, but future work hopes to investigate how the bioactivities of honey may be able to be retained after UHT or pasteurisation, or when incorporated into baking *etc*. In the meantime, there are other means of sterilising honey (including gamma-irradiation), and it could be that honey may need to be added to foods at a later stage in the development process to avoid any loss of function. These points would need to be addressed by any food manufacturer wishing to add honey to their products to improve health.

The results of this thesis do demonstrate, though, that honey could have positive effects on certain aspects of human health, including improved weight regulation, immunity, lipid levels and cognitive performance without having to be consumed at excessively high levels. Certainly, it appears that honey does not need to replace all the sugar ingested per day, and it could be that the use of honey in a supplemental form or as a partial substitute may be adequate to confer a particular effect. Consumption of food in human populations is driven not only by the need to fulfill our body's requirements for energy and nutrients, but also by emotional and psychological cues. Many people eat not only when they are hungry, but when they are sad or lonely. Similarly, effective advertising can induce a person to purchase and consume a food product that they did not necessarily want or need. Our abilities to control our dietary intake are therefore controlled by a multitude of different factors, and it is unlikely that many individuals would have the willpower required to refrain from eating products that contained sugars, whilst consuming only those that contained honey. However, most sugar is consumed in manufactured products, and dramatic changes in dietary sugar intake could be affected if food manufacturers switched to using honey in their products. It would also be of interest in future studies to investigate exactly how much honey must be consumed to have a specific effect *in vivo*, and whether honey is more effective when taken as a supplement to the typical diet (thereby increasing the overall sugar intake) or as a partial substitute.

As well, it would be of interest to determine for how long honey must be consumed before health benefits are observed. In the present studies,

improvements in weight regulation were seen after short-term feeding (see Chapters 5 and 6), but corresponding data for brain function was limited (see Chapter 10). Data from the EPM maze (assessing anxiety), for example, showed no interaction of diet x time for the duration of time spent in the open arm of the maze (indicating less anxiety), although there was a significant reduction in anxiety in honey-fed rats overall. A closer look at the one-way ANOVAs from each time point of this study revealed that honey-fed rats were only significantly less anxious compared with those fed sucrose at the 9-month testing session (see Figure 10.8). This would suggest that at least 9 months of consuming honey is therefore required to observe this effect. However, analysis of the 6-month data shows that that the mean duration of time spent in the open arm of the maze was at least twice as high in honey-fed rats compared with those fed sucrose at this testing session also (27.1 *vs* 12.4 seconds). This did not reach statistical significance because of the high data variability, but it is possible that significant differences may have been observed if larger sample sizes had been used. If this is the case, it could be speculated that honey may be able to be consumed for a shorter period of time than initially thought to exert its effects on cognitive ability and/or mental acuity.

It is important to recognise, though, that feeding honey to rodents for 6 or 9 months is not equivalent to a human individual consuming it for the same period of time. The life span of a Sprague Dawley rat is only 2–3 years when kept in a laboratory setting (Hubert *et al*., 2000), thus 6–9 months is ¼–½ of its life span. By comparison, the average life expectancy in US populations is 77.9 years (Minino, 2005). Potentially, an individual may therefore need to consume honey for at least 20 years of his or her life to have an effect on cognitive performance, although it is also unknown whether the outcomes might differ depending on when the honey was consumed (*i.e.* in early life, in later later, or sporadically throughout the life-time). Such questions would need to be addressed in future studies.

11.2.1 Negative Aspects of Consuming Honey

Although the positive aspects of consuming honey have been addressed thoroughly in this study (see Chapter 1), there is some evidence to suggest that consuming honey, particularly in higher quantities, can also be detrimental for some individuals. Recent research has demonstrated, for example, that some honeys are derived from plants containing pyrrolizidine alkaloids, these substances being known to cause poisoning, liver damage and death in humans,

as well as cancer in rodent models (reviewed by Edgar *et al*., 2002). It has been suggested that pyrrolizidine alkaloids occur in approximately 3% of the world's flowering plants (Smith and Culvenor, 1981), but that the occurrence of honeys containing these compounds may be considerably higher, as several of these plant species are specifically grown for other uses (*e.g. Borago officinalis* and *Echium* spp. are high in alpha- and gamma-linoleic acids, making them good seed oils; Roeder *et al*., 2001). Honeybees are used to pollinate the flowers of these plants in such areas, however, and honey is considered to be a valuable byproduct of this industry (Edgar *et al*., 2002). As mentioned earlier, daily consumption of honey per capita ranges from 0.0–1.3 grams per person, and individuals in European countries consume the highest amounts (World Health Organisation, 1998). It has been suggested that certain individuals (*i.e.* those living in areas where plants containing these compounds are known to grow abundantly) may therefore be consuming up to 7.8 μg of pyrrolizidine alkaloids per day, this being significantly higher than the 0.1 μg/day specified as safe by world authorities (World Health Organisation, 1998). Caution against consuming large quantities of honey is therefore advised for floral sources that are either unrecognised, or known to contain these substances.

Intake of honey (particularly in large amounts) may also be detrimental to some individuals because of the high levels of fructose ingested. In the present studies it has been suggested that the fructose in the honey may be a better alternative to sucrose or glucose in the diet as it results in a lower GI (Samanta *et al*., 1985). However, GI measures only the glucose response and not that resulting from the intake of fructose, as fructose metabolism follows a completely different pathway from that of glucose (Elliot *et al*., 2002). In addition, fructose has been shown to have the opposite effect of glucose on insulin, leptin and ghrelin, three hormones that are known to have key roles in the regulation of food intake (Wylie-Rosett *et al*., 2004), such that a high fructose intake may actually result in an *increase* in overall food intake because of decreased satiety. Such findings were not corroborated in the studies presented in this thesis, though, the animals being fed honey generally consuming the same amount of food as those fed sucrose or a sugar-free diet. High-fructose diets have also been shown to induce fatty liver in rats and ducks, and to increase hepatic lipid peroxidation in rats (reviewed by Gaby, 2005). Similar findings have not been detailed in human studies, although one small study has reported that the fructose moiety of sucrose may have been responsible for the liver injury seen in healthy non-obese

males who consumed 20–35% of calories as sucrose for 30 days (Porikos and Van Itallie, 1979).

Furthermore, ingestion of honey has been shown to have a laxative effect in some people because of its high fructose content (Ladas *et al.,* 1995; Ladas and Raptis 1999). Fructose is generally poorly absorbed from the gut, and the unabsorbed sugar can act to draw water into the bowel because of its osmotic load, thereby leading to diarrhoea, bloating, flatus and abdominal discomfort (Elliott *et al*., 2002). However, glucose has been shown to enhance the absorption of fructose, and the ratio of glucose:fructose present can be a key factor in determining its effects on the gut in susceptible individuals (Ament *et al*., 1997). In addition, the laxative properties of honey may actually aid some individuals, particularly those suffering from constipation.

In both human and animal studies, fructose consumption has also been shown to elevate triglyceride levels (reviewed by Elliott *et al*., 2002). This was also not seen in the studies undertaken in this thesis, but this is likely because the levels of fructose consumed did not reach those used in the published studies. Indeed, whilst honey is considered to be a high-fructose food (the fructose content of honey is ~45%), the levels of fructose ingested by the rats in the present studies rarely exceeded 5% in the diet overall. The impact of consuming honey related to high fructose levels may therefore be minimal in human consumption, except in cases where large quantities are ingested over a short period of time.

11.3 CONCLUSIONS

Although there are a few potential negative consequences of adding honey to the daily diet, its use should not be completely disregarded. In 2003, four United Nations agencies, (including the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO)) commissioned a report that stated that the total of free sugars (all monosaccharides and disaccharides added to foods by manufacturers, cooks or consumers, plus sugars naturally present in honey, syrups and fruit juices) should not account for more than 10% of the energy-intake of a healthy diet. But, despite this, intake of sugars (particularly sucrose, glucose and high fructose corn syrups) continues to increase in Western countries (Joint FAO/WHO Expert Consultation, 2003).

The ability to at least replace some of the more commonly used sugars with a product that could positively influence health has to therefore be a step in the right direction. However, with the burden that chronic diseases are now

placing on our global society, education as to the role that diet, nutrition and lifestyle can play is also key. Recent changes in the world's food economy has resulted in shifting dietary patterns, including increased consumption of energy-dense diets high in fat, particularly saturated fat, and low in unrefined carbohydrates (Joint FAO/WHO Expert Consultation, 2003). Importantly, these patterns, along with an increase in sedentary lifestyle are all contributing to the escalating prevalance of chronic diseases such as Diabetes, cardiovascular disease and cancer in Western populations. Food manufacturers must therefore take some level of responsibility for the nutritional status of the foods they produce, rather than being driven purely by levels of profit that can be achieved.

In conclusion, it appears that the consumption of honey may offer several advantages to human health, specifically as a possible replacement for other sugars in the diet. In particular, honey appears to have a significant impact on weight regulation, with reductions in weight gain being observed after only a few weeks of dietary change. Importantly, these reductions in weight gain also continued to be observed in older age (after long-term feeding), when it is well accepted that it is generally harder to manage weight because of a reduction in metabolic rate. Unfortunately, despite the fact that three studies were undertaken to investigate the effects of honey on weight gain, no definitive conclusions as to how it induced these effects were able to be elucidated. Based on the data in this thesis, it appears that the reduced weight gain seen in honey-fed animals is not due to satiety as food intake was generally not reduced in these animals compared with those fed sucrose. It is possible that the honeys used in this thesis were lower GI than the sucrose, and that this could have accounted for some of the differences, but this too is questioned as rats fed mixed sugars as in honey did not display the same reductions in weight. The data does suggest that there are substances present in the non-sugar fraction of honey that are responsible for the weight loss effects seen. What these substances are is currently unknown, as are the actions by which they could be exhibiting these effects. More research into this area is needed to better understand how honey is having this effect and what concentrations are required to be eaten to affect weight gain.

Importantly, long-term feeding of honey also resulted in a significant reduction in blood glucose levels suggesting that it may be a good sugar source for those who are susceptible to Diabetes or insulin resistance. As well, honey demonstratably increased HDL-cholesterol (the "good" cholesterol) compared with an equivalent amount of dietary sucrose, suggesting that consumption of

honey may also help to reduce the risk of cardiovascular heart disease in some individuals (low levels of HDL-cholesterol are a key indicator of heart disease).

Although full analyses were not undertaken, preliminary data also suggested that honey may help improve immunity in later life. As declining immune function is one of the key driving forces behind the development of many age-related disorders, this may also act to reduce the cost of medical care, whilst improving an individuals quality of life.

Finally, consumption of honey appears to have a significant impact on some aspects of cognition in later life, possibly helping to maintain brain function. Cognitive decline is a complex issue, and much more work needs to be done in this area to better elucidate these effects. The use of different behavioural tasks, as well as brain analyses, for example, may generate new data that could help researchers better understand what effects honey can have on maintaining brain function in older age.

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Appendix 1:

AC-350 Automatic Calorimeter Instruction Manual.

Supplied by Felicity Jackson, Manager, Nutrition Laboratory, Institute of Food, Nutrition & Human Health, Massey University

Appendix 2:

Raw Data from the Behaviour Experiments

(Chapter 10)

Raw Elevated Plus Maze data (mean ± SD) from the 3, 6, 9 and 12 month testing sessions for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the testing session = 300 seconds)

	3 Months			6 Months			9 Months			12 Months		
Behaviour	**Sugar Free**	**Sucrose [a]**	**Honey**	**Sugar Free**	**Sucrose [a]**	**Honey**	**Sugar Free [b]**	**Sucrose [a]**	**Honey**	**Sugar Free**	**Sucrose**	**Honey[c]**
# Animals tested	14	14	14	14	14	15	14	14	15	13	14	14
Total arm entries	9.7 ± 5.9	11.5 ± 5.8	12.1 ± 1.7	7.1 ± 3.8	8.5 ± 4.3	10.3 ± 4.8	9.6 ± 4.3	9.9 ± 4.9	12.0 ± 5.4	6.9 ± 3.7	5.5 ± 3.3	9.7 ± 4.8
Open arm entries	1.4 ± 1.6	2.2 ± 1.6	2.5 ± 2.4	1.3 ± 1.5	1.1 ± 1.3	2.5 ± 2.0	2.5 ± 2.0	2.2 + 2.0	4.3 ± 2.6	1.6 ± 1.8	0.8 ± 1.3	2.4 ± 2.3
Closed arm entries	8.4 ± 4.6	9.3 ± 4.4	9.6 ± 3.6	5.9 ± 2.7	6.9 ± 3.5	7.8 ± 3.1	7.1 ± 2.6	8.3 ± 2.5	7.7 ± 3.5	5.2 ± 2.3	4.7 ± 2.5	7.3 ± 2.8
# Entering open arm	8/14	10/13	9/14	7 / 14	7 / 13	13 / 15	10/11	9/13	13/15	8/13	6/14	9/12
% Open arm time	6.5 ± 8.0	9.8 ± 7.9	13.2 ± 12.5	5.6 ± 7.7	4.1 ± 5.9	9.0 ± 6.9	10.9 ± 10.6	8.3 ± 8.0	21.7 ± 15.0	7.2 ± 8.7	3.2 ± 5.8	8.1 ± 8.4
% Closed arm time	66.4 ± 30.1	65.0 ± 11.2	63.1 ± 22.7	56.9 ± 27.0	59.5 ± 26.5	54.2 ± 24.6	61.4 ± 24.1	66.1 ± 18.2	40.8 ± 27.8	57.7 ± 15.4	61.9 ± 25.7	55.4 ± 29.1
% Centre area time	13.8 ± 10.3[†]	24.1 ± 9.0	17.3 ± 8.1	37.4 ± 22.3	35.2 ± 26.4	30.5 ± 19.9	27.7 ± 21.3	25.6 ± 13.6	24.2 ± 10.8	35.1 ± 11.8	30.5 ± 23.6	35.9 ± 25.7
Duration open arm time (secs)	2.1 ± 2.7	3.3 ± 2.6	4.4 ± 4.1	16.9 ± 23.1	12.4 ± 17.8	27.1 ± 20.6	32.7 ± 31.8	24.8 ± 24.0	65.2 ± 45.0	21.7 ± 26.2	9.4 ± 17.5	24.3 ± 25.1
Latency to enter open arm [d]	162.5 ± 133.4	126.8 ± 123.1	134.0 ± 131.8	191.2 ± 123.3	183.0 ± 124.0	109.8 ± 110.7	98.4 ± 99.9	153.1 ± 112.3	64.8 ± 102.2	154.8 ± 127.8	214.6 ± 111.2	120.0 ± 122.0
Mean number rears	4.0 ± 3.2	5.4 ± 3.1	5.7 ± 4.3	1.8 ± 1.8	1.4 ± 1.4	1.2 ± 1.6	1.6 ± 1.2	1.4 ± 1.5	1.4 ± 2.0	4.2 ± 2.1	2.7 ± 1.7	5.7 ± 2.2
Total number rearing events	56	70	84	26	20	18	17	15	22	56	39	68
Total unprotected head dips	0.8 ± 1.4	1.6 ± 1.5	2.1 ± 4.3	0.7 + 1.2	0.9 ± 1.8	1.2 ± 1.5	1.2 ± 1.2	1.5 ± 1.7	3.9 ± 3.4	0.8 ± 1.1	0.4 ± 0.7	0.8 ± 1.1
Total protected head dips	1.2 ± 1.9	2.1 ± 1.9	0.8 ± 0.9	1.8 ± 1.9	1.2 ± 1.6	1.9 ± 1.7	2.2 ± 2.0	2.3 ± 1.8	3.0 ± 1.7	1.6 ± 1.1	0.9 ± 1.4	1.6 ± 1.6
Total protected head peeps	9.4 ± 5.5	8.6 ± 3.7	9.3 ± 3.8	5.2 ± 4.4	6.2 ± 5.8	6.6 ± 4.5	7.9 ± 4.3	9.7 ± 3.4	7.0 ± 5.2	5.5 ± 3.9	7.1 ± 4.9	7.7 ± 5.0
Total protected half body out	3.0 ± 3.5	4.4 ± 2.2	3.4 ± 1.8	5.4 ± 3.5	5.2 ± 3.5	4.2 ± 3.3	3.2 ± 1.5	3.8 ± 2.5	5.1 ± 3.1	6.4 ± 3.2	4.2 ± 3.0	6.3 ± 3.7
# falling off EPM	0	1	0	0	1	0	3	1	0	0	0	2

[a] n=13 as one animal fell from the open arm and was not included in the data
[b] n=11 due to animals falling off the maze not being included in the data
[c] n=12 due to animals falling off the maze not being included in the data
[d] animals not entering the open arm were given a latency time of 300 sec (the duration of the test)

Raw Y Maze data (mean ± SD) from the 3, 6, 9 and 12 month testing sessions (0–60 second time bin only) for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the full testing session = 300 seconds).

	3 Months			6 Months			9 Months			12 Months [c]		
Behaviour	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**
# Animals tested	14	14	15	14	14	13 [b]	14	13 [b]	13 [b]	10	10	9 [b]
Duration Start arm (secs)	13.3 ± 10.7	14.6 ± 14.0	15.0 ± 17.3	6.4 ± 4.4	9.4 ± 7.0	12.2 ± 10.9	16.3 ± 11.6	17.5 ± 10.8	8.6 ± 7.0	13.4 ± 11.3	15.2 ± 12.7	12.3 ± 10.3
Duration Novel arm (secs)	25.3 ± 9.7	22.9 ± 12.9	26.4 ± 10.8	15.5 ± 11.4	15.4 ± 11.0	22.6 ± 11.2	9.0 ± 12.0	5.6 ± 7.7	10.6 ± 11.0	23.7 ± 11.5	12.7 ± 13.5	14.5 ± 9.4
Duration Other arm (secs)	16.4 ± 8.9	15.4 ± 4.9	15.0 ± 7.4	16.6 ± 13.3	9.4 ± 9.3	9.4 ± 9.8	22.9 ± 12.2	17.9 ± 6.6	27.3 ± 14.0	6.9 ± 10.2	15.5 ± 18.2	24.7 ± 13.8
Duration in centre area (secs)	12.8 ± 5.2	11.7 ± 6.3	11.7 ± 7.9	18.5 ± 10.8	25.8 ± 10.6	15.8 ± 10.2	11.8 ± 6.2	19.0 ± 7.1	13.4 ± 7.1	16.0 ± 7.3	16.6 ± 11.2	8.5 ± 12.0
% time in Start arm (secs)[a]	28.3 ± 21.2	25.1 ± 28.2	29.7 ± 30.4	21.5 ± 17.1	27.8 ± 19.0	28.1 ± 22.5	33.9 ± 23.4	42.3 ± 23.6	18.3 ± 14.3	29.2 ± 21.5	38.6 ± 33.4	28.7 ± 24.0
% time in Novel arm [a]	38.6 ± 22.6	38.7 ± 24.8	37.7 ± 22.8	38.7 ± 25.8	45.2 ± 25.4	52.2 ± 19.5	47.5 ± 23.5	44.8 ± 18.2	58.3 ± 28.1	54.5 ± 25.5	28.4 ± 27.4	53.8 ± 29.9
% time in Other arm [a]	35.2 ± 17.8	34.6 ± 12.6	32.2 ± 14.4	39.8 ± 25.6	27.0 ± 26.6	19.7 ± 17.2	18.6 ± 23.6	12.9 ± 14.6	23.4 ± 23.3	16.4 ± 23.3	33.0 ± 32.4	17.5 ± 22.0
Number of visits to Start arm	1.6 ± 0.5	2.0 ± 0.8	16. ± 0.5	1.1 ± 0.6	1.9 ± 0.6	1.6 ± 0.8	1.7 ± 0.6	2.1 ± 0.5	1.4 ± 0.5	1.7 ± 0.5	1.7 ± 0.5	1.8 ± 0.5
Number of visits to Novel arm	1.3 ± 0.8	1.5 ± 0.8	1.4 ± 0.8	1.1 ± 0.7	1.5 ± 0.8	1.4 ± 0.5	1.2 ± 0.6	1.9 ± 0.6	1.2 ± 0.4	1.3 ± 0.5	0.9 ± 0.7	1.4 ± 0.5
Number of visits to Other arm	1.6 ± 0.7	1.9 ± 0.5	1.7 ± 0.8	1.6 ± 0.5	1.6 ± 0.7	0.9 ± 0.4	0.7 ± 0.6	1.1 ± 0.6	0.8 ± 0.6	0.7 ± 0.8	0.8 ± 0.6	0.5 ± 0.5
Latency to leave start arm (secs)	7.9 ± 10.9	8.5 ± 11.0	15.7 ± 34.8	6.1 ± 15.0	2.6 ± 2.8	4.1 ± 7.9	5.7 ± 8.5	2.3 ± 2.5	6.0 ± 7.4	2.2 ± 2.9	4.4 ± 5.9	1.9 ± 2.4
Latency to visit novel arm (secs)	6.9 ± 18.7	8.4 ± 17.6	11.5 ± 37.1	19.4 ± 34.2	21.4 ± 28.9	7.2 ± 8.1	17.4 ± 19.9	6.1 ± 4.5	10.4 ± 7.4	6.8 ± 6.9	29.7 ± 50.7	15.6 ± 15.6

[a] Percentage time spent in a particular arm were calculated exclusive of the time spent in the centre area

[b] Animals were excluded from the data analysis if they did not leave the start arm for the duration of the testing session, or if they spent > 75% of their time in the centre area.

[c] Only 10 animals per treatment performed the task at the 12 month testing session

Raw Y Maze data (mean ± SD) from the 3, 6, 9 and 12 month testing sessions (0–120 second time bin only) for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the full testing session = 300 seconds).

	3 Months			6 Months			9 Months			12 Months [c]		
Behaviour	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**
# Animals tested	14	14	15	14	14	14 [b]	14	13 [b]	13 [b]	10	10	10
Duration Start arm (secs)	29.1 ± 20.2	28.1 ± 16.5	29.9 ± 32.2	20.4 ± 11.3	24.1 ± 15.5	21.7 ± 19.6	36.2 ± 18.5	34.2 ± 24.2	33.2 ± 18.1	21.9 ± 18.1	27.7 ± 25.3	29.0 ± 14.6
Duration Novel arm (secs)	38.3 ± 14.1	39.7 ± 20.2	43.7 ± 26.0	23.2 ± 17.6	35.6 ± 20.0	44.4 ± 28.3	31.6 ± 19.3	32.4 ± 13.1	34.8 ± 18.8	38.4 ± 13.8	29.5 ± 29.1	42.5 ± 30.3
Duration Other arm (secs)	30.5 ± 14.1	31.9 ± 13.8	32.1 ± 17.1	34.7 ± 28.8	19.8 ± 14.7	26.2 ± 28.0	19.0 ± 17.7	16.8 ± 12.9	24.8 ± 26.7	27.8 ± 25.6	36.3 ± 35.3	21.0 ± 11.6
Duration in centre area (secs)	27.1 ± 10.4	26.6 ± 11.3	22.0 ± 15.8	37.5 ± 23.8	40.6 ± 11.7	27.6 ± 19.4	33.1 ± 18.8	36.6 ± 13.0	27.2 ± 12.0	31.9 ± 19.5	26.6 ± 15.3	27.5 ± 14.6
% time in Start arm (secs)[a]	31.6 ± 21.1	25.7 ± 20.8	28.8 ± 28.8	29.3 ± 19.1	30.6 ± 19.2	25.9 ± 20.4	41.7 ± 16.6	39.8 ± 22.5	40.1 ± 18.2	24.7 ± 18.0	30.5 ± 28.9	32.4 ± 20.0
% time in Novel arm [a]	33.7 ± 19.1	37.7 ± 13.0	36.9 ± 23.5	31.7 ± 21.7	44.9 ± 23.5	49.8 ± 27.0	36.6 ± 17.2	40.1 ± 16.9	41.1 ± 16.5	45.7 ± 20.2	32.4 ± 29.6	44.8 ± 27.1
% time in Other arm [a]	33.1 ± 14.5	36.2 ± 16.3	32.6 ± 16.1	39.0 ± 26.1	24.5 ± 18.1	24.2 ± 21.6	21.7 ± 18.9	20.1 ± 15.0	18.8 ± 19.6	29.6 ± 27.7	37.2 ± 30.9	22.8 ± 10.7
Number of visits to Start arm	2.3 ± 0.5	2.7 ± 1.1	2.1 ± 0.7	2.3 ± 0.7	2.5 ± 0.9	1.9 ± 1.0	2.5 ± 0.7	2.7 ± 0.8	2.1 ± 0.5	1.9 ± 0.7	1.9 ± 0.6	2.3 ± 0.7
Number of visits to Novel arm	2.0 ± 1.0	2.3 ± 1.5	1.8 ± 1.0	1.6 ± 0.9	2.3 ± 1.3	1.9 ± 0.8	1.4 ± 0.7	2.5 ± 0.8	1.7 ± 0.8	1.6 ± 0.5	1.5 ± 1.0	1.5 ± 0.5
Number of visits to Other arm	2.3 ± 1.0	2.8 ± 0.7	2.2 ± 1.0	1.4 ± 0.9	1.7 ± 1.0	1.3 ± 0.5	1.2 ± 0.9	1.7 ± 0.8	1.0 ± 0.7	1.2 ± 0.9	1.4 ± 1.0	1.3 ± 0.7

[a] Percentage time spent in a particular arm were calculated exclusive of the time spent in the centre area

[b] Animals were excluded from the data analysis if they did not leave the start arm for the duration of the testing session, or if they spent > 75% of their time in the centre area.

[c] Only 10 animals per treatment performed the task at the 12 month testing session

Raw Y Maze data (mean ± SD) from the 3, 6, 9 and 12 month testing sessions (0–180 second time bin only) for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the full testing session = 300 seconds).

	3 Months			6 Months			9 Months			12 Months [c]		
Behaviour	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**
# Animals tested	14	14	15	14	14	14 [b]	14	13 [b]	13 [b]	10	10	10
Duration Start arm (secs)	41.1 ± 20.0	40.3 ± 17.3	38.5 ± 36.8	28.4 ± 20.1	45.5 ± 28.3	37.5 ± 28.1	60.7 ± 19.3	62.5 ± 29.4	52.6 ± 23.5	45.8 ± 21.7	38.6 ± 34.6	48.4 ± 28.1
Duration Novel arm (secs)	54.5 ± 20.9	56.5 ± 19.5	57.5 ± 41.7	50.9 ± 27.6	45.0 ± 21.1	68.8 ± 43.4	42.5 ± 25.6	38.3 ± 12.7	54.5 ± 23.9	45.2 ± 15.5	52.0 ± 49.5	61.8 ± 47.1
Duration Other arm (secs)	48.5 ± 25.5	49.6 ± 15.5	51.5 ± 28.0	46.3 ± 37.8	34.7 ± 24.4	34.7 ± 40.3	32.1 ± 26.8	25.8 ± 19.8	40.3 ± 32.1	38.9 ± 29.6	52.7 ± 54.1	30.3 ± 21.8
Duration in centre area (secs)	41.1 ± 19.3	44.0 ± 10.4	37.2 ± 20.4	50.3 ± 25.2	54.8 ± 17.6	39.0 ± 27.0	44.6 ± 22.5	53.4 ± 18.5	32.5 ± 11.4	50.1 ± 24.6	36.8 ± 21.8	39.5 ± 27.4
% time in Start arm (secs)[a]	30.3 ± 13.7	29.6 ± 13.0	29.9 ± 24.1	24.1 ± 17.9	36.4 ± 20.8	30.3 ± 21.6	43.9 ± 19.7	48.5 ± 20.1	38.2 ± 18.1	34.3 ± 14.6	28.5 ± 26.9	35.6 ± 19.5
% time in Novel arm [a]	33.8 ± 15.4	35.4 ± 12.6	34.9 ± 25.1	41.8 ± 22.1	35.6 ± 15.7	50.5 ± 25.2	31.4 ± 16.9	31.5 + 13.1	39.3 ± 15.5	36.8 ± 19.9	36.7 ± 32.0	42.2 ± 27.5
% time in Other arm [a]	34.7 ± 15.9	36.6 ± 12.3	34.3 ± 17.0	34.1 ± 23.9	28.0 ± 19.3	19.0 ± 13.7	24.7 ± 20.0	20.0 ± 14.3	22.5 ± 20.3	28.9 ± 22.5	34.8 ± 31.0	22.2 ± 14.0
Number of visits to Start arm	2.9 ± 0.8	3.3 ± 1.2	2.5 ± 1.0	2.5 ± 0.8	3.1 ± 1.0	2.3 ± 1.2	2.7 ± 0.8	3.1 ± 0.6	2.4 ± 0.7	2.6 ± 0.8	2.3 ± 0.8	2.5 ± 0.8
Number of visits to Novel arm	2.5 ± 1.1	2.9 ± 1.5	2.1 ± 0.9	2.1 ± 0.7	2.9 ± 1.9	2.3 ± 1.0	1.9 ± 0.9	2.9 ± 1.0	2.0 ± 0.7	2.0 ± 0.7	1.9 ± 1.0	2.0 ± 0.8
Number of visits to Other arm	2.1 ± 1.1	2.5 ± 0.7	2.1 ± 0.9	1.7 ± 1.1	2.4 ± 1.5	1.4 ± 0.5	1.6 ± 1.1	2.1 ± 0.8	1.5 ± 1.0	1.5 ± 0.7	1.5 ± 1.1	1.4 ± 0.9

[a] Percentage time spent in a particular arm were calculated exclusive of the time spent in the centre area

[b] Animals were excluded from the data analysis if they did not leave the start arm for the duration of the testing session, or if they spent > 75% of their time in the centre area.

[c] Only 10 animals per treatment performed the task at the 12 month testing session

Raw Y Maze data (mean ± SD) from the 3, 6, 9 and 12 month testing sessions (0–300 second time bin only) for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the full testing session = 300 seconds).

	3 Months			6 Months			9 Months			12 Months [c]		
Behaviour	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**
# Animals tested	14	14	15	14	14	14 [b]	14	13 [b]	13 [b]	10	10	10
Duration Start arm (secs)	66.4 ± 31.6	67.5 ± 43.1	67.0 ± 60.5	62.8 ± 46.1	84.3 ± 65.2	75.5 ± 37.7	91.1 ± 42.7	91.4 ± 48.2	95.2 ± 52.2	82.1 ± 38.7	73.4 ± 49.9	98.7 ± 73.1
Duration Novel arm (secs)	54.8 ± 44.6	50.6 ± 34.8	57.6 ± 66.9	85.5 ± 49.2	65.6 ± 23.9	89.8 ± 44.5	70.5 ± 53.1	74.0 ± 27.2	96.4 ± 65.7	81.6 ± 44.4	83.4 ± 63.1	104.2 ± 90.1
Duration Other arm (secs)	83.2 ± 46.3	82.4 ± 39.3	82.3 ± 41.7	59.0 ± 39.6	68.8 ± 55.2	77.8 ± 65.6	67.8 ± 51.1	53.8 ± 38.2	60.7 ± 44.8	57.4 ± 46.5	84.2 ± 88.7	36.4 ± 23.4
Duration in centre area (secs)	67.6 ± 30.2	70.7 ± 16.7	64.4 ± 34.6	87.2 ± 39.7	81.2 ± 38.3	65.1 ± 40.3	70.6 ± 30.7	80.8 ± 23.2	47.6 ± 16.7	78.9 ± 37.5	59.0 ± 34.6	60.7 ± 49.9
% time in Start arm (secs)[a]	29.5 ± 11.6	31.5 ± 17.5	29.9 ± 23.4	29.9 ± 18.3	37.3 ± 24.9	32.7 ± 17.2	40.3 ± 20.2	43.6 ± 20.4	38.4 ± 20.4	37.4 ± 17.1	32.5 ± 24.5	41.2 ± 26.3
% time in Novel arm [a]	35.3 ± 17.5	34.0 ± 14.3	37.9 ± 23.0	42.5 ± 21.2	31.0 ± 12.2	43.9 ± 21.3	29.5 ± 18.1	32.9 ± 15.6	38.1 ± 23.8	35.6 ± 16.8	34.6 ± 23.5	42.2 ± 31.7
% time in Other arm [a]	35.6 ± 17.2	34.9 ± 15.4	34.5 ± 16.2	27.6 ± 16.2	31.7 ± 23.1	23.4 ± 10.9	30.2 ± 23.3	23.5 ± 16.4	23.5 ± 19.4	27.0 ± 24.2	32.9 ± 29.7	16.6 ± 11.3
Number of visits to Start arm	3.8 ± 1.1	4.2 ± 1.5	3.4 ± 1.1	3.4 ± 0.9	3.5 ± 1.0	3.0 ± 1.5	3.3 ± 1.0	3.4 ± 0.7	2.8 ± 0.8	3.1 ± 0.7	3.1 ± 1.1	3.0 ± 1.1
Number of visits to Novel arm	3.5 ± 1.5	3.7 ± 2.0	3.0 – 1.1	3.0 ± 1.2	3.5 ± 2.0	3.0 ± 1.2	2.3 ± 1.1	3.9 ± 0.7	2.5 ± 0.8	2.7 ± 0.7	2.6 ± 1.5	2.6 ± 1.3
Number of visits to Other arm	2.4 ± 1.4	2.7 ± 1.1	2.2 ± 1.1	2.5 ± 1.5	3.1 ± 1.9	2.3 ± 1.1	2.2 ± 1.2	2.6 ± 1.1	2.0 ± 1.1	2.4 ± 2.2	2.1 ± 1.5	1.8 ± 1.3

[a] Percentage time spent in a particular arm were calculated exclusive of the time spent in the centre area

[b] Animals were excluded from the data analysis if they did not leave the start arm for the duration of the testing session, or if they spent > 75% of their time in the centre area.

[c] Only 10 animals per treatment performed the task at the 12 month testing session

Raw Open Field data (mean ± SD) from the first half of the 6 and 12 month testing sessions (0–150 seconds) for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the full testing session = 300 seconds).

	6 Months			12 Months		
Behaviour	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**
# Animals tested	10	10	10	12	12	12
Duration Centre zone (secs)	1.9 ± 1.2	1.1 ± 1.0	1.0 ± 0.9	1.1 ± 0.7	2.5 ± 3.0	2.3 ± 1.8
Duration Middle Zone (secs)	11.2 ± 6.5	13.1 ± 7.5	7.2 ± 5.4	8.4 ± 9.8	19.6 ± 23.1	11.9 ± 10.4
Duration Outside Zone (secs)	136.6 ± 6.8	130.0 ± 23.1	141.7 ± 5.9	140.5 ± 10.3	127.6 ± 25.4	135.8 ± 11.9
# Visits to centre zone	1.6 ± 0.5	1.2 ± 0.4	1.4 ± 1.0	1.1 ± 0.4	1.4 ± 0.7	1.1 ± 0.4
# Visits to middle zone	5.0 ± 2.7	4.6 ± 1.7	3.5 ± 2.5	2.1 ± 1.5	3.0 ± 2.3	2.3 ± 2.0
# Visits to outside zone	4.4 ± 2.4	4.3 ± 1.8	3.3 ± 2.0	1.9 ± 1.1	2.6 ± 1.9	2.1 ± 2.0
# Line crosses in middle zone	2.6 ± 1.9	2.6 ± 2.5	2.3 ± 2.6	1.1 ± 1.7	1.5 ± 2.2	1.6 ± 2.2
# Line crosses in outside zone	33.9 ± 15.3	34.9 ± 15.5	43.8 ± 8.8	24.7 ± 11.9	13.2 ± 10.0	17.0 ± 5.2
Total moves[a]	47.4 ± 18.9	47.6 ± 54.1	54.1 ± 14.0	31.0 ± 15.3	21.7 ± 10.4	24.1 ± 7.6

[a] Includes all line crosses and zone entries

Raw Open Field data (mean ± SD) from the second half of the 6 and 12 month testing sessions (151–300 seconds) for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the full testing session = 300 seconds).

	6 Months			12 Months		
Behaviour	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**
# Animals tested	10	10	10	12	12	12
Duration Centre zone (secs)	1.5 ± 2.9	0.18 ± 0.5	1.5 ± 3.9	0.1 ± 0.3	0.0 ± 0.0	0.14 ± 0.4
Duration Middle Zone (secs)	7.6 ± 7.2	3.2 ± 4.7	7.8 ± 7.9	7.7 ± 5.0	8.3 ± 18.5	7.1 ± 9.4
Duration Outside Zone (secs)	139.9 ± 9.6	145.7 ± 5.0	139.7 ± 10.3	141.2 ± 5.1	140.7 ± 18.5	141.8 ± 9.5
# Visits to centre zone	0.6 ± 1.0	0.4 ± 0.3	0.4 ± 0.5	0.1 ± 0.4	0.0 ± 0.0	0.1 ± 0.4
# Visits to middle zone	2.1 ± 2.4	0.9 ± 1.2	2.0 ± 1.7	1.9 ± 1.2	0.4 ± 0.7	0.6 ± 1.2
# Visits to outside zone	2.6 ± 1.5	1.8 ± 1.0	2.5 ± 1.4	2.7 ± 1.0	1.3 ± 0.5	1.5 ± 0.9
# Line crosses in middle zone	2.9 ± 2.8	0.4 ± 0.9	3.1 ± 2.7	2.7 ± 3.3	0.3 ± 0.5	0.6 ± 1.1
# Line crosses in outside zone	22.3 ± 18.6	23.2 ± 16.2	23.8 ± 10.4	17.6 ± 7.2	10.8 ± 10.0	11.8 ± 9.0
Total moves[a]	30.4 ± 24.3	26.4 ± 17.1	31.8 ± 11.5	25.0 ± 10.0	12.9 ± 9.8	14.6 ± 10.4

[a] Includes all line crosses and zone entries

Raw Open Field data (mean ± SD) from the full 300 seconds of the 6 and 12 month testing sessions for animals fed a sugar-free diet, 7.9% sucrose or 10% honey (duration of the full testing session = 300 seconds).

	6 Months			12 Months		
Behaviour	**Sugar-Free**	**Sucrose**	**Honey**	**Sugar-Free**	**Sucrose**	**Honey**
# Animals tested	10	10	10	12	12	12
Duration Centre zone (secs)	3.5 ± 2.6	1.3 ± 1.4	2.5 ± 4.5	1.2 ± 0.8	2.5 ± 3.0	2.1 ± 1.8
Duration Middle Zone (secs)	18.8 ± 7.6	16.2 ± 11.0	15.0 ± 12.4	16.1 ± 12.4	28.1 ± 40.2	18.1 ± 17.9
Duration Outside Zone (secs)	277.5 ± 8.6	276.6 ± 15.1	282.4 ± 15.2	282.7 ± 13.2	269.1 ± 42.5	279.8 ± 19.3
# Visits to centre zone	2.1 ± 1.1	1.3 ± 0.7	1.8 ± 1.4	1.3 ± 0.8	1.4 ± 0.7	1.1 ± 0.4
# Visits to middle zone	7.1 ± 3.8	5.4 ± 2.6	5.5 ± 4.0	4.0 ± 2.5	3.3 ± 2.6	3.1 ± 2.7
# Visits to outside zone	6.0 ± 2.9	5.1 ± 2.4	4.9 ± 3.2	3.7 ± 1.9	3.0 ± 2.1	3.0 ± 2.9
# Line crosses in middle zone	5.4 ± 3.0	3.0 ± 3.1	5.4 ± 5.2	3.9 ± 4.3	1.8 ± 2.5	2.6 ± 3.2
# Line crosses in outside zone	56.3 ± 32.5	58.2 ± 28.9	66.6 ± 13.6	42.3 ± 16.2	24.0 ± 18.8	30.9 ± 9.0
Total moves[a]	77.0 ± 41.4	73.1 ± 29.7	84.1 ± 19.4	55.1 ± 23.5	33.4 ± 18.8	40.8 ± 12.5

[a] Includes all line crosses and zone entries

Raw Object Recognition data (mean ± SD) from Phase 1 (3 minutes, animals were presented with two identical objects) and Phase 2 (3 minutes, animals were presented with a novel object and a copy of that presented in Phase 1) for animals fed a sugar-free diet, 7.9% sucrose or 10% honey.

	Diet		
Behaviour	**Sugar Free**	**Sucrose**	**Honey**
Number of animals assessed [a]	8	8	9
Phase 1			
# Visits to object 1	4.0 ± 1.2	2.3 ± 1.4	2.8 ± 1.6
# Visits to object 2	3.4 ± 2.3	2.7 ± 1.9	2.4 ± 1.0
Total visits	7.4 ± 2.5	5.0 ± 2.8	5.2 ± 2.2
Duration spent exploring object 1	15.5 ± 5.3	9.0 ± 4.5	16.9 ± 6.0
Duration spent exploring object 2	11.5 ± 7.5	10.1 ± 7.3	11.1 ± 4.6
Total time exploring	27.0 ± 9.1	19.1 ± 8.1	28.0 ± 9.0
Latency to visit object 1	48.7 ± 51.3	53.9 ± 54.9	60.4 ± 44.8
Latency to visit object 2	29.7 ± 14.1	45.7 ± 50.1	76.8 ± 56.3
Phase 2			
# Visits to object 3	2.1 ± 0.8	2.3 ± 0.9	1.0 ± 1.0
# Visits to novel object	3.8 ± 2.1	3.6 ± 1.8	2.7 ± 1.3
Total visits	5.9 ± 2.1	5.9 ± 2.2	3.7 ± 1.6
Duration spent exploring object 3	6.3 ± 4.9	6.6 ± 2.8	4.6 ± 1.9
Duration spent exploring novel object	25.5 ± 15.5	28.6 ± 21.1	26.2 ± 12.8
Total time exploring	31.8 ± 18.4	35.2 ± 21.4	29.3 ± 12.3
Latency to visit object 3	48.6 ± 42.0	28.8 ± 22.8	65.4 ± 28.8
Latency to visit novel object	20.8 ± 22.8	16.9 ± 15.2	10.8 ± 8.5

[a] 10 animals from each treatment performed the task. Animals were excluded from the data analysis if they didn't explore one or more objects in either phase.

Raw Object Recognition data (mean ± SD) from the Light Extinction task for animals fed a sugar-free diet, 7.9% sucrose or 10% honey. Animals were presented with an active lever that turned the lights off for 30 seconds and an inactive lever which had no effect.

	Diet		
Behaviour	**Sugar Free**	**Sucrose**	**Honey**
Number of animals assessed	10	10	10
Total 20 Minute Session			
Number of active lever presses	23.5 ± 8.7	30.1 ± 17.8	28.4 ± 14.4
Number of inactive lever presses	12.8 ± 8.7	18.5 ± 9.7	11.9 ± 6.0
Total number of lever presses	36.3 ± 13.9	48.6 ± 26.2	40.3 ± 17.9
AL_{on}/AL_{tot} ratio [a]	0.47 ± 0.11	0.45 ± 0.16	0.48 ±0.11
0–10 Minutes			
Number of active lever presses	13.6 ± 5.6	15.6 ± 11.0	15.5 ± 6.8
Number of inactive lever presses	7.9 ± 5.3	13.1 ± 8.0	8.3 ± 4.6
Total number of lever presses	21.5 ± 7.9	28.7 ± 17.2	23.8 ± 9.5
AL_{on}/AL_{tot} ratio [a]	0.55 ± 0.20	0.51 ± 0.22	0.47 ± 0.21
11–20 Minutes			
Number of active lever presses	9.9 ± 4.5	12.7 ± 8.8	10.6 ± 10.4
Number of inactive lever presses	4.5 ± 4.2	5.1 ± 2.6	3.9 ± 2.5
Total number of lever presses	14.4 ± 7.8	17.8 ± 10.0	14.5 ± 12.3
AL_{on}/AL_{tot} ratio [a]	0.46 ± 0.09	0.50 ± 0.27	0.50 ± 0.21

[a] The number of Active lever presses while the lights were on (AL_{on}) as a proportion of the total Active lever presses (Al_{tot}). A value of 0.5 indicates that the animals did not discriminate between pressing the active lever while the lights were on or off, suggesting that the rats did not learn that pressing the active lever while the lights were OFF have no effect.

Appendix 3:

Published Papers from this Thesis.

The Effect of Honey Compared to Sucrose, Mixed Sugars, and a Sugar-Free Diet on Weight Gain in Young Rats

L.M. CHEPULIS

ABSTRACT: To determine whether honey, sucrose, and mixed sugars as in honey have different effects on weight gain, 40 6-wk-old Sprague-Dawley rats were fed a powdered diet that was either sugar free or contained 8% sucrose, 8% mixed sugars as in honey, or 10% honey freely for 6 wk. Weight gain and food intake were assessed weekly, and at completion of the study blood samples were removed for measurement of blood sugar (HbA1c) and a fasting lipid profile. The animals were then minced and total percentage body fat and protein measured. Overall percentage weight gain was significantly lower in honey-fed rats than those fed sucrose or mixed sugars, despite a similar food intake. Weight gains were comparable for rats fed honey and a sugar free diet although food intake was significantly higher in honey-fed rats. HbA1c and triglyceride levels were significantly higher in all sugar treatments compared with rats fed a sugar free diet, but no other differences in lipid profiles were reported. No differences in percentage body fat or protein levels were reported.

Keywords: honey, obesity, sugars, weight gain

Introduction

Obesity is a major concern for westernized populations, and in the USA alone over 50% of all adults are now considered to be overweight or obese (Flegel and others 1998). Surveys consistently show that adults are concerned by weight and its health-related implications, and that most individuals are making a concerted effort to either maintain or lose weight (Serdula and others 1999; Scott and others 2006). Despite this, however, the end result is that the number of overweight and obese individuals has increased steadily over the last 30 y.

The increase in weight observed in western society is a complex issue, although reasons include factors such as increased workloads/stress and reduced levels of exercise. However, the largest contributor may be the change in diet that has occurred in the last quarter century. Today, convenience and "junk" foods are readily available, and they often constitute a large proportion of the weekly food intake. These foods are generally highly processed, low in nutrition, and often contain excessively high levels of sugar and fat.

Whereas food companies continue to produce, and successfully market, products that are high in both sugar and fat, there is now a public-driven trend toward the development of food products that are healthier and/or confer specific health benefits. In recent years, a large number of foods and beverages have become available with a "diet" version using artificial sweeteners such as aspartame and saccharin (Anderson and McMurray 1998). These substances are generally considered safe and nontoxic (Leon and others 1989; Ellwein and Cohen 1990; Renwick 1993; Butchko and others 2002); although limited evidence is producing conflicting data. Several authors, for example, have reported a higher incidence of bladder cancer in rats and mice fed sodium saccharin (Bryan and others 1970; Schoenig and others 1985; Ellwein and Cohen 1990; Rewick 1990; Garland and Cohen 1995). In addition, ingestion of aspartame has been linked with migraines/headaches (Lipton and others 1989; van den Eeden and others 1994; Schiffman 1995), muscle tremors, vision problems, and mental confusion (Anon. 1994).

An alternative to the use of artificial sweeteners is the replacement of sugar with less energy-dense alternatives and the use of sugars other than sucrose and glucose as sweetening agents. Honey is a naturally occurring sweetener that contains a mix of both simple and complex sugars, as well as vitamins, minerals, acids, and enzymes (Molan 1996). Furthermore, it has a lower glycemic index (GI) than sucrose (Samanta and others 1985), and the beneficial effects of consuming honey are well established in the literature. These include improved antioxidant capacity (Taormina and others 2001; Gheldof and others 2003; Schramm and others 2003), enhanced gut motility (Ladas and Raptis 1999), enhanced cytokine production (Tonks and others 2003), and a prebiotic effect (Sanz and others 2005; Ezz El-Arab and others 2006). In addition, anecdotal evidence suggests that ingested honey may have the potential to enhance absorption from the gut.

This trial was therefore designed to ascertain what effect honey might have on the weight gain of young, growing rats, as compared with those fed a sucrose diet or a diet containing sugars in the same proportion of honey. A 4th treatment group, fed a sugar-free diet, was also included in this trial as a control.

Materials and Methods

Experimental design

Forty Sprague-Dawley rats, aged approximately 6 wk at the start of the trial, were sourced from the small animal research facility at AgResearch, Hamilton, New Zealand. The trial was carried out in the Animal Behaviour Research Facility (Building BL10) at Waikato Univ., Hamilton, New Zealand. The research room was maintained

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doi: 10.1111/j.1750-3841.2007.00286.x

at 22 ± 1 °C with a 12-h light/dark cycle (lights on 0700 h). This study was approved by the Waikato Univ. Animal Ethics Committee.

Experimental diets

A honeydew honey (*Northofagus solandrii*) with a known high level of hydrogen peroxide production (antibacterial activity due to peroxide equivalent to 23.4% phenol equivalent) and a high antioxidant content (TEAC = 2.7 mmol/L) was sourced from Tom Penrose, North LongBurn, Rangiora, New Zealand. A sugar profile of this honey was determined using in-house GLC methods by Agriquality (Auckland, New Zealand) and was used to determine the concentrations of sugars added to the mixed sugar diet (diet nr 3) such that the concentrations of the various sugars were the same as in the honey diet.

The diets were prepared such that they approximated the composition of a typical New Zealand diet. Based upon data from the 1997 Natl. Nutrition Survey (New Zealand Ministry of Health 1999), the diets were formulated so that of 100% total energy, 15% to 16% came from protein, 35% came from fat, and 45% to 47% came from carbohydrate.

Four experimental diets were prepared by Crop and Food Research, Palmerston North, New Zealand, to contain no sugar (diet nr 1), 8% sucrose (diet nr 2), 8% mixed sugars (diet nr 3), or 10% honey (diet nr 4). All diets were prepared to contain a minimum of 4% water. An additional 20 mL of water was also added to each kilogram of the nonhoney diets to account for the fact that the honey contained 20% water (measured using a refractometer). Diets were prepared no more than 10 d prior to commencing the study and were kept in the dark at 4 °C or –15 °C for the duration of the study. Standard rodent vitamin and mineral mixes was prepared to comply with the requirements for growth as specified by the Natl. Research Council (1995). The composition of the diets is given in Table 1.

Subsamples of the 4 diets were analyzed by the Nutrition Laboratory at Massey Univ., Palmerston North, New Zealand, for measurement of energy (bomb calorimetry). The percentage dry matter of these samples was also determined at the start and end of the trial by drying preweighed samples at 105 °C for 16 h.

Table 1—Composition of experimental diets (g/kg)

Ingredient	Sugar free (diet nr 1)	Sucrose (diet nr 2)	Mixed sugars (diet nr 3)	Honey (diet nr 4)
Lactalbumin[a]	160	160	160	160
Corn oil[b]	150	150	150	150
Sucrose[c]	–	80	0.5	–
Fructose[c]	–	–	40.9	–
Glucose[c]	–	–	30.1	–
Maltose[c]	–	–	3.4	–
Maltodextrin[b]	–	–	5.1	–
Honey	0	0	0	100
Starch	525	445	445	445
Mineral mix[e]	50	50	50	50
Vitamin mix[f]	5	5	5	5
Water	60	60	60	40
Energy (kJ/g)	18.02	18.21	18.74	17.82

[a] Alatal 825, New Zealand Milk Products.
[b] Davis Trading Co., Palmerston North, New Zealand.
[c] Sigma Chemical Co.
[d] Avicel PH102, Commercial Minerals Ltd, Auckland, New Zealand.
[e] A mixture supplying: (g/kg diet) Ca 6.29, Cl 7.79, Mg 1.06, P 4.86, K 5.24, Na 1.97; (mg/kg diet) Cr 1.97, Cu 10.7, Fe 424, Mn 78.0, Zn 48.2; (μg/kg diet) Co 29.0, Mo 152, Se 151.
[f] A mixture supplying: (mg/kg diet) retinol acetate 5.0, DL-α-tocopherol acetate 200.0, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-panothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg/kg diet) ergocalciferol 25.0, cyanocobalamin 50.0.

Experimental procedures

Animals were weighed upon receipt, and those within this starting weight range (140 to 150 g) were randomly allocated to one of the 4 dietary treatments. The remaining animals were housed in standard rat cages with plastic bottoms and metal grid tops (final measurements 45 × 25 × 30 cm high), with access to rodent pellets and water. These animals were weighed each morning and added into the trial once at the appropriate weight.

During the experiment, the rats were housed in standard rat cages (2 animals per cage) that had an 8 mm metal grid floor sitting 2 cm above the cage bottom. Blotting paper was laid on the bottom of the cage (below the mesh) to absorb the urine and collect the spilled diet and faeces. A 25 cm long piece of 90 mm PVC tubing was affixed into each cage using a metal hose clamp. This allowed the animals to get off the mesh floor.

Glass jars containing the appropriate diet were weighed at the start of each week and placed into the respective cages. New food was weighed and added to each food pot as required (no more than once daily) so that food was available freely.

Every 7 d, the rats were weighed and the existing food pots removed and weighed. The blotter papers were removed and allowed to dry for a minimum of 48 h at 26 °C, and each pair of rats was transferred to a cleaned cage with new weighed food. Fresh water was provided at all times, with water bottles cleaned twice weekly. Once dry, the spilled diet was scraped off the blotters and separated from the faeces/hair by passing through a 2 mm sieve and weighed.

On day 42, all rats were anaesthetized using CO_2 gas. Samples (3 to 5 mL) of blood were taken from the heart of each animal via cardiac puncture using 19 gauge needles. Approximately 1.5 mL of the removed blood was added to an EDTA blood collection tube and the remainder added to a standard vacutainer. These latter blood samples were allowed to clot, before centrifuging at 3000 × *g* for 10 min and removing the serum. Both the EDTA-collected blood and the serum were then analyzed at Waikato Hospital (Hamilton, New Zealand) using standard laboratory procedures for measurement of glycated haemoglobin (HbA1c) (HPLC Biomate Affinity column) and fasting lipid profiles (Roche Method using P 800 Hitachi). All animals with euthanized with CO_2 gas after the blood removal, and the bodies numbered and stored in a freezer for later analysis.

After thawing, each rat was minced using a Sunmile SM-G50 mincer (Vantage Systems, Ltd.). The samples were double bagged and frozen before sending to the nutrition laboratory at Massey Univ. for measurement of total body fat by Soxhlet fat extraction (AOAC 991.36) analyses. The rat samples were also analyzed for % protein (using a nitrogen–protein conversion factor of 6.25) by the LECO total combustion method (AOAC 968.06).

Statistical analyses

Analysis of variance (ANOVA) was carried out on endpoints. All analyses were performed using Genstat statistical software (GenStat 2003).

Results

Two rats (both in the honey treatment group) had noticeable quantities of blood in their urine from day 32 onward. These 2 rats were segregated from the other animals; but as they appeared to be otherwise healthy and eating well, they were not excluded from the trial. Urine samples were collected on day 40 from both animals and, at completion of the study, both animals were taken to Vet Pathology at MedLab, Hamilton, New Zealand, for a full postmortem. No particular cause could be found to explain the cause of the bleeding, other than the presence of chronic cystitis in both rats. No infections were present in the urine or bladder, and it was

concluded that the blood in the urine was likely to be incidental and not related to treatment. For this reason the data pertaining to these 2 animals were included in the statistical analyses. Data and statistical findings are given in Table 2.

The results of this study suggest that there are beneficial effects of replacing sugar with honey in the diet (see Table 2). In particular, overall percentage weight gain in honey-fed rats was similar to that observed in rats fed a sugar-free diet, and was significantly reduced, by 16.7%, compared with sucrose-fed rats after 6 wk (see Figure 1). Figure 2 shows the mean percentage weekly weight gains for all 4 treatments.

Total food and calorie intake was significantly higher in all sugar treatments compared with the sugar-free treatment group. However, no statistically significant differences in total food intake were observed between any of the 3 sugar treatments. Similarly, HbA1c levels were significantly lower in rats fed the sugar-free diet (4.02 mmol/L) than in rats fed the other 3 diets (4.09 to 4.14 mmol/L), and there were no differences observed between the 3 sugar treatments. In addition, no differences in total-, LDL-, or HDL-cholesterol or triglyceride levels were observed between any of the treatments after 6 wk of feeding.

Total percentage body fat and protein levels were not statistically different between treatments, although variability in body fat measurements were high (coefficients of variations were 31.5% to 39.4% for the 3 sugar treatment groups). The animals were not skinned prior to mincing, and the testing laboratory noted that because the ground rat samples contained large amounts of skin and hair, it was hard to get a representative sample from each animal (sampling requires only 1 g). Total percent protein levels were comparable for all 4 treatments.

Discussion

As the results of this study demonstrate, the type of sugars that are ingested as a part of the daily diet can have a significant impact on levels of food intake and weight gain during free feeding. Overall percentage weight gain, for example, was significantly higher in rats fed the mixed sugars- and sucrose-containing diets compared with those fed the sugar free diet. This may be accounted for by the increased kilojoule intake, although research is equivocal. Kanarek and Orthen-Gambill (1982) demonstrated that animals given access to 32% solutions of sucrose or fructose in addition to their standard diet gained more weight per calorie consumed than animals given only the standard (sugar free) diet after 50 d. In contrast, a number of other researchers have reported that weight gains in rodents are still higher with sucrose even when matched with caloric-equivalent starch diets (Reiser and Hallfrisch 1977; Hill and others 1980; Hallfrisch and others 1981; Morris and others 2003). However, animal age has also been shown to be a major contributing factor. Adult rats given sucrose displayed increased body weight after 1 to

Table 2—Endpoint measurements for rats fed the experimental diets for 8 wk in a weight evaluation study

Result	Unit	Sugar free (diet nr 1)	Sucrose (diet nr 2)	Mixed sugars (diet nr 3)	Honey (diet nr 4)	LSD	*P*-value	Degrees freedom
Overall weight gain	%	139.7 ± 8.6[f]	155.0 ± 6.5[e]	151.5 ± 14.4[e]	138.3 ± 11.2[f,g]	10.38	0.009	16
Overall food intake[a,b]	g/6 w	1643 ± 62	1824 ± 63[e]	1762 ± 47[e]	1772 ± 77[e]	84.6	0.003	16
Kiljoules consumed[a]	kJ/6 w[c]	26090 ± 1116	29103 ± 874[e]	30337 ± 1144[e]	28597 ± 1376[e]	1343	0.000	19
HbA1c[c]	mmol/L	4.02 ± 0.12	4.14 ± 0.07[e]	4.09 ± 0.11[e]	4.11 ± 0.19[e]	0.0777	0.026	15
Total cholesterol[c]	mmol/L	2.83 ± 0.62	2.69 ± 0.59	2.34 ± 0.36	2.66 ± 0.34	0.319	0.532	15
LDL cholesterol[c]	mmol/L	0.31 ± 0.18	0.2 ± 0.16	0.09 ± 0.09	0.18 ± 0.18	0.1850	0.079	15
HDL cholesterol[c]	mmol/L	2.09 ± 0.46	2.1 ± 0.43	1.93 ± 0.3	2.07 ± 0.18	0.4096	0.803	15
Triglycerides[c]	mmol/L	0.93 ± 0.30	0.9 ± 0.24	0.74 ± 0.38	0.83 ± 0.28	0.2849	0.797	15
Total body fat	%	7.8 ± 1.2	8.8 ± 3.5	9.5 ± 3.6	8.8 ± 2.8[d]	NR	0.627	37
Total protein	%	20.9 ± 0.6	20.9 ± 1.3	20.6 ± 1.0	20.5 ± 1.0[d]	NR	0.844	37

HbA1c = glycated haemoglobin; HDL = high density lipoprotein; LDL = low density lipoprotein.
[a]Food and energy intake is assessed per cage (that is, per 2 animals). As such, there are only 5 units per treatment.
[b]Food intake corrected for percentage dry matter (determined by drying duplicate samples for 16 h at 105 °C).
[c]Due to an inability to collect sufficient blood from all animals, $n = 9$, 10, 7, and 8 for rats fed sugars free, sucrose, mixed sugars, and honey diets, respectively.
[d]$n = 8$ only, as 2 animals from this treatment group had blood in their urine and were investigated by Vet Pathology.
[e]Significant compared to sugar-free diet.
[f]Significant compared to sucrose diet.
[g]Significant compared to mixed sugars diet.

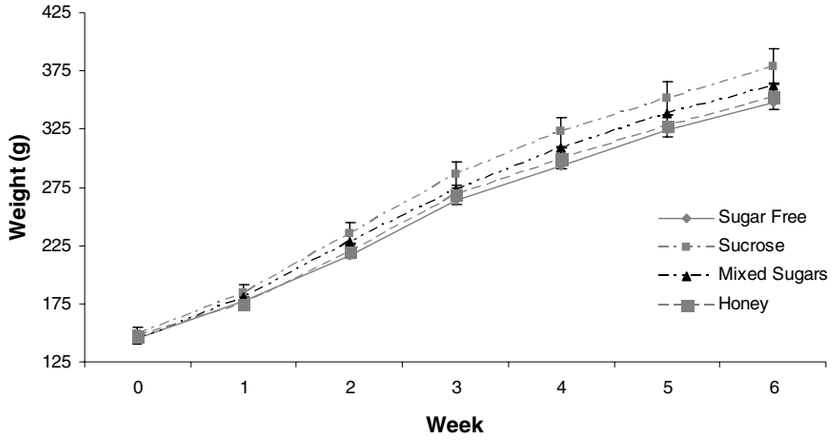

Figure 1—Actual weight gains for rats fed diets containing either no sugar (diet nr 1), 8% sucrose (diet nr 2), 8% mixed sugars (diet nr 3), or 10% honey (diet nr 4). Diets nr 1, 2, and 3 had 20 mL of water added per kilogram of diet such that the moisture contents were the same in all 3 diets.

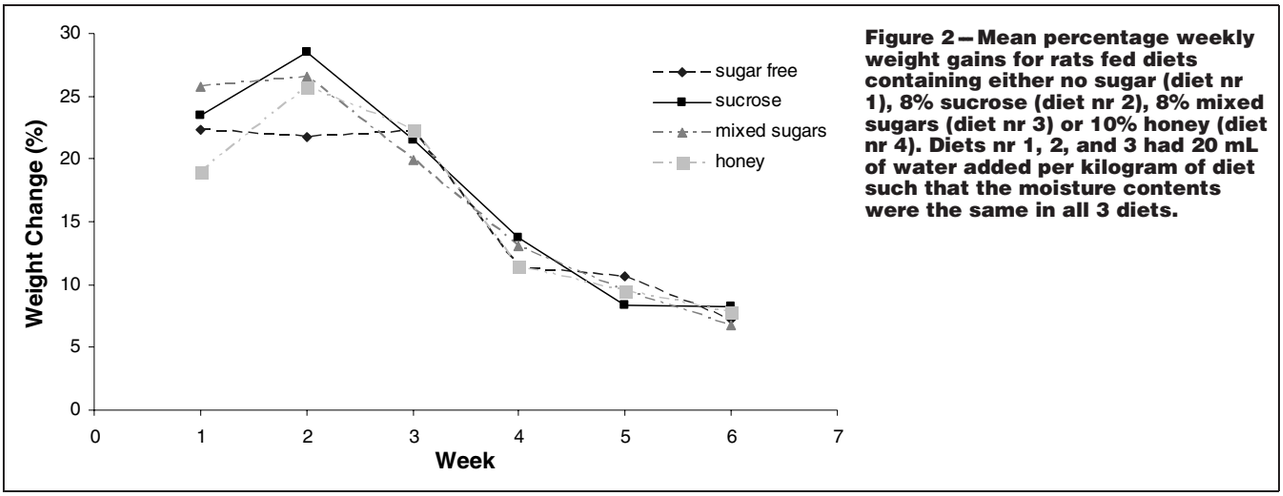

Figure 2—Mean percentage weekly weight gains for rats fed diets containing either no sugar (diet nr 1), 8% sucrose (diet nr 2), 8% mixed sugars (diet nr 3) or 10% honey (diet nr 4). Diets nr 1, 2, and 3 had 20 mL of water added per kilogram of diet such that the moisture contents were the same in all 3 diets.

2 wk (Kanarek and Hirsch 1977; Kratz and Levitsky 1979) whereas weanling rats given sugar consumed more calories than their sugar-free counterparts, but with little measurable differences in weight gain/kilojoule until they reached 10 to 12 wk of age (Kanarek and Marks-Kaufmann 1979). The animals in this study were only 6 wk old at the start of the study, weighing approximately 140 g. At this young age, these animals could easily have been diverting all extra energy into growth rather than fat storage. This would likely have resulted in increased percentage body protein levels though and no differences were observed between any treatments. However, an unpublished study in our laboratory has demonstrated that rats fed a diet containing 60% honey (of which 12% is water) had significantly higher fecal nitrogen output and significantly reduced protein digestibility compared with animal fed equivalent amounts of either sucrose or mixed sugars as in honey. The results of this study therefore suggest that protein absorption and utilization may be reduced in honey-fed rats, although the levels of sugars and honey used in the diets of this unpublished study are approximately 5 times higher than those used in the present study. It could be possible therefore that the young animals used in this study were diverting more energy into protein synthesis and growth but that body protein levels increased at a slower rate than their sucrose or mixed sugars counterparts. As the percentage body fat data in this study were inconclusive, it is impossible to know whether the presence of sucrose or mixed sugars in the diet led to increased body fat levels. However, dietary carbohydrates have been shown to accelerate fat synthesis by increasing the capacity of the lipogenic pathway (Morris and others 2003).

Importantly, the increased weight gain observed with the mixed sugar-fed rats did not occur in the honey-fed rats even though both treatments had similar food/energy intakes. Limited research has speculated that honey can have a laxative effect *in vivo* (Ladas and others 1995; Ladas and Raptis 1999) as honey contains 35% to 45% fructose (White 1975) and incomplete fructose absorption has been shown to have this effect in healthy subjects (Ravich and others 1983; Truswell and others 1988; Rumessen 1992). However, this is likely not to be responsible for the decreased weight gains observed in honey-fed rats in this study, as fructose only constituted 4.1% of the total honey diet and it is doubtful that such concentrations would have a laxative effect. Furthermore, any fructose-related decreases in absorption/weight gain should also have been observed in rats fed the mixed sugars diet and this did not occur. Fecal output was not assessed in this study, so we cannot conclusively demonstrate that the rate of gut absorption/transit was affected in rats fed honey or mixed sugars. However, unpublished work from our laboratory suggests that fecal output does not differ significantly between rats fed 8% sucrose, 10% honey, or sugar free diets after 10 wk of feeding.

In addition, the sucrose, mixed sugars, and honey diets all contained the same level of fructose, but the fructose to glucose ratio varied. Sucrose is a dimer consisting of 1 glucose molecule attached to 1 fructose molecule; therefore the ratio of fructose to glucose is 1:1. In the honey used in this trial (and in the mixed sugars diet also) the ratio of fructose to glucose was 4:3. This agrees with the levels of fructose and glucose reported in other honeys (White 1975, 1980). A limited number of studies have demonstrated that the presence of glucose can facilitate and improve absorption of fructose and vice versa (Truswell and others 1988; Shiota and others 1998, 2002). Therefore, it is possible that the differing fructose:glucose ratios in the different diets may have played a role. Possible differences in absorption may also have resulted in differences in glycemic control. Fructose and honey have both been shown to have lower glycemic index values than sucrose (Shambaugh and others 1990), and it is possible that honey-fed rats have more stable blood sugar levels. This, in turn, may lead to better metabolism and decreased amino acid catabolism and fat deposition. However, this potential sugar-related effect was also not observed in rats fed the mixed sugars diet.

One explanation is the presence of hydrogen peroxide in the honey, as research has shown hydrogen peroxide to be a strong insulin-mimetic agent (Czech and others 1974; Hayes and Lockwood 1987; Heffetz and others 1990). Levels of hydrogen peroxide production in honey vary depending on floral source, and so on, but were shown to be in the range of 0.5 to 3.5 mmol/L in high peroxide-producing honeys (Bang and others 2003). Although not measured using the same method, the honeydew honey used in this study has also been shown in our laboratory to have a similar level of hydrogen peroxide production. These levels agree with the 1 to 3 mmol/L shown to induce tyrosine phosphorylation (an insulin-mimetic effect) *in vitro* (Hayes and Lockwood 1987; Heffetz and others 1990), although hydrogen peroxide levels in honey would arguably be considerably less *in vivo* after dilution in the gut.

Limited clinical studies have demonstrated that honey, sucrose, and fructose do have differential effects blood glucose levels (Samanta and others 1985; Al-Waili 2004). In both studies, plasma glucose levels were significantly lower in both healthy and diabetic patients who had consumed honey (20 to 75 g) than in those who had consumed equivalent amounts of dextrose, sucrose, or fructose

after 1 to 3 h. It is possible therefore that the ingestion of honey in this study may have resulted in decreased plasma glucose levels, thereby resulting in reduced fat deposition. Whilst there appears to be no evidence in the literature to demonstrate that hydrogen peroxide is absorbed from the gut or that the levels produced are sufficient to trigger insulin receptors, this study does demonstrate that there is something present in the nonsugar component of honey that results in decreased weight gain per kilojoule. It is possible that the honey may have had a physiological effect on the stomach. Goldschmidt and Burkert (1955) have reported that honey acts as if it contains acetylcholine and choline esters, which could affect the gastric muscle. Other hormonal/neurotransmitter activities are also possible as a result of consuming honey, although nothing has been reported in the literature to support this.

Al Waili (2004) also observed that whereas a single dose of dextrose (glucose) or artificial honey (35 g dextrose + 40 g fructose in 250 mL) led to increases in cholesterol and triglycerides over 1 to 3 h, the effect was not observed with honey. In this same study, daily feeding of honey for 15 d decreased cholesterol by 8%, LDL-C by 11%, and C-reactive protein by 57% in patients with elevated cholesterol compared with baseline values. No effects on total cholesterol, LDL-C, HDL-C, or triglyceride levels were observed in this study with any of the 4 treatments; however, due to difficulties with blood sampling we were unable to collect sufficient blood from all animals to allow 10× replicates to be undertaken for each treatment. As well, the variation for all cholesterol parameters was high for all treatments, and this may have masked any significant differences that may have been present. It is unknown whether this variability is due to the method of collection, the inherent interanimal variation, or the assay employed.

Housing animals in pairs is not ideal for weight investigation studies, and this may have affected the results obtained. O'Connor (2000) has demonstrated that food intake in rats is higher when animals are housed in pairs compared with when they are housed individually. When housed in pairs 1 animal may dominate over the other and consume more food. Fighting did occur in the cages at times, and particular cages did show a discrepancy for the weights of the 2 animals on certain weigh-in dates (despite similar weights the week before). However, a look at the complete 6-wk weight data did not show any patterns for reduced weight gain for a given animal or for ongoing weight discrepancies for any of the cages.

Conclusions

Rats fed a honey-containing diet showed significantly reduced weight gains after only 6 wk compared with those fed other sugars. High intakes of sugars are associated with weight gain and obesity. The replacement of sugars (including sucrose and fructose) with honey in particular foods and beverages may therefore provide sweetness without additional calorie-induced weight gain.

Acknowledgments

This research was funded by Fonterra Co-operative Group Ltd., New Zealand, as a part of a PhD program. The author would also like to thank Dr. Peter Molan and Dr. Judi McWhirter, both of Waikato Univ., for their assistance.

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Journal of Complementary and Integrative Medicine

Volume 4, *Issue* 1 2007 *Article* 8

The Effects of Honey Compared With Sucrose and a Sugar-free Diet on Neutrophil Phagocytosis and Lymphocyte Numbers after Long-term Feeding in Rats

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The Effects of Honey Compared With Sucrose and a Sugar-free Diet on Neutrophil Phagocytosis and Lymphocyte Numbers after Long-term Feeding in Rats*

Lynne M. Chepulis

Abstract

To determine whether honey and sucrose would have differential effects on levels of neutrophil phagocytosis after long-term feeding 36 2-month old Sprague Dawley rats were fed a powdered diet that was either sugar-free or contained 7.9% sucrose or 10% honey (honey is 21% water) *ad libitum* for 52 weeks. The percent of neutrophils exhibiting phagocytosis, and the percentage of leukocytes that were lymphocytes were then measured by flow cytometry after 52 weeks. Results: Neutrophil phagocytosis was similar between sucrose- and honey-fed rats, and lower in rats fed the sugar-free diet (79.2%, 74.7% and 51.7 %, respectively). The percentage of leukocytes that were lymphocytes differed significantly between all three treatments, the levels being highest in honey-fed rats (53% vs 40.1% and 29.5% for sucrose- and sugar-free fed rats). In conclusion: Honey may have a beneficial effect on immune activity, possibly attenuating the decline seen in older age.

KEYWORDS: Honey, sucrose, neutrophil phagocytosis, immunity, lymphocytes

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*This research was funded by Fonterra Brands Ltd, New Zealand as a part of a PhD program. The author would also like to thank Dr Peter Molan and Dr Nicola Starkey of Waikato University for their assistance.

INTRODUCTION

Immune function is known to decline in older age and limited data suggests that this is due to dysregulation of the immune system rather than a decrease in immune response (Lesourd, 1997). In particular, aging in humans is associated with a decrease in cell-mediated immunity including a reduction in peripheral blood lymphocyte counts, and with changes in T lymphocyte subpopulations (Lesourd, 1997; Castle, 2000). Similarly, neutrophil activity has also been shown to decline in older populations (Wenisch et al. 2000; Butcher et al. 2001). The latter may be of particular importance as neutrophils are considered to be the "first line of defense", these being the leukocytes that respond most rapidly to a pathogenic invasion. Research has shown that prebiotics have the ability to enhance immune responses (Yamada et al. 1999; Schley and Field, 2002) and anecdotal evidence suggests that honey can stimulate immune function. Limited research has demonstrated that honey can stimulate the release of tumour necrosis factor-α •and cytokines in monocyte cells (Tonks et al., 2001, 2003), and increase proliferation of B- and T-lymphocytes and neutrophils *in vitro* (Abuharfeil et al. 1999). In addition, honey has been demonstrated to stimulate antibody production during primary and secondary immune responses against thymus-dependent and thymus-independent antigens (Al-Waili and Haq, 2004). This trial was therefore designed to ascertain what effect honey might have on the immunity levels of older rats, compared with those fed a sucrose-based or sugar-free diet, after long term feeding.

MATERIALS AND METHODS

Experimental Animals and Housing

This study included 36 male Sprague Dawley rats, aged approximately 8 weeks at the start of the trial, sourced from the small animal research facility at AgResearch, Hamilton, New Zealand. The trial was carried out in the Behaviour Research Facility at Waikato University, Hamilton, New Zealand.

The research room was maintained at 22±1°C with a 12 hour light/dark cycle (lights off 0700h). This study was approved by the Waikato University Animal Ethics Committee.

Diets

Three experimental diets were prepared to contain either no sugar (diet # 1), 7.9% sucrose (diet # 2) or 10% Honeydew honey (diet # 3) (the honey was 21% water) as well as the following (g/kg diet): skim milk powder, 95; casein, 120; oil, 160; cellulose, 50; mineral mix, 50, sugar-free vitamin mix, 5, starch, 365. The diets were prepared such that they were equivalent to a typical New Zealand diet, based upon data from the 1997 National Nutrition Survey (1999). Of 100% total energy, 15–16% came from protein, 35% came from fat, and 45–47% came from carbohydrate. Low GI amylose was used in place of sucrose and honey in the sugar-free diet.

Diets were prepared monthly and were kept in the dark at 4°C or -15°C for the duration of the study. Standard rodent vitamin and mineral mixes was prepared as described previously (Chepulis, 2007).

Experimental Design

All animals were weighed upon receipt, randomly allocated to one of three experimental diets and housed individually in standard rat cages (45 cm x 25 cm x 30 cm high) with plastic bottoms and metal grid lids. Food jars containing the appropriate diet were placed into the respective cages and new diet added to the jars every two days so that food was available *ad libitum*. Water was freely available, and replaced twice weekly. All animals were weighed every 1–2 weeks.

On days 364 and 365 (half of each treatment group on each day), the rats were anaethetised using CO_2 gas. Each animal underwent a cervical dislocation before a sample of blood was removed from the heart via cardiac puncture using 19 gauge needles. The samples were taken to AgResearch, Hamilton, New Zealand (within 2 hours of collection) and assayed using Flow cytometry as follows. Briefly, 100 µl of whole blood was added to pre-cooled FACS tubes in an ice bath. A sample (50 µl) of fluorescein isothiocyanate (FITC)-labelled *E.coli* (prepared in-house using non-oponised K12 strain *E.coli* and Fluorescein conjugate W-2861) containing 4 x 10^6 bacteria was then added to each tube (except the control) and the tubes incubated at 37°C for 10 minutes (the control tube was left in the icebath). After incubation, 50 µl of FITC-labelled *E.coli* was added to the control tube. All tubes were then lysed with FACS lyse buffer (BD Biosciences Catalog number 349202) and the tubes incubated at room temperature in the dark for 10 minutes. The tubes were then centrifuged at 1200 rpm for 10 minutes at 18°C, the supernatant discarded and the pellets re-suspended in 1mL each of PBS buffer. The tubes were again centrifuged as described and the supernatant discarded. Each sample was then re-suspended in 500 µl running buffer (1% paraformaldehyde in PBS), and 50 µl Trypan blue (0.4% in PBS) added to each tube. These samples were then run on a Becton Dickenson Flow Cytometer and the percent neutrophil phagocytosis and percent leukocytes that were lymphocytes determined.

Statistical Analyses

All values are expressed as mean ± SD. A one-way analysis of variance (ANOVA) was performed on all parameters to determine if there were statistical differences between the three dietary treatments.

RESULTS

All animals appeared healthy throughout the 52-week study and no adverse events were recorded. The results are presented in Table 1. Mean overall percentage weight gain in honey-fed rats was similar to that observed in rats fed a sugar-free diet, and was significantly reduced compared with sucrose-fed rats after 52 weeks ($p = 0.015$). Mean overall percentage weight

gain was also significantly reduced in rats fed the sugar-free diet compared with the sucrose-fed rats ($p = 0.004$). Food intake did not differ between the three dietary treatments throughout the 12 month period (see Chepulis and Starkey, 2007 for further details).

The percentage of neutrophils that exhibited fluorescent phagocytosis was similar between sucrose- and honey-fed rats, and both of these treatments were significantly higher than rats fed the sugar-free diet (both $p < 0.0001$). The percentage of leukocytes that were lymphocytes was nearly twice as high in honey-fed rats as in those fed the sugar free diet ($p < 0.0001$), and nearly 13% higher than the sucrose-fed rats ($p = 0.002$). The percentage lymphocyte values for rats fed the sucrose and sugar-free diets were significantly different at $p < 0.0001$.

Table 1: Endpoint Data (mean ± SD) for rats fed diets that were either sugar-free, or contained 7.9% sucrose or 10% honey for 12 months.

Result	Unit	Diet		
		Sugar-free (Diet # 1)	**Sucrose (Diet # 2)**	**Honey (Diet # 3)**
Overall weight gain	%	102.5 ± 19.7†	130.6 ± 26.7*	107.2 ± 13.8 †
Overall food intake[a]	g/7 w	1246.4 ± 85	1243.6 ± 111	1244.8 ± 89
Overall kilojoule intake	kJ/7 w	23182 ± 1580	23019 ± 2053	22730 ± 1620
Neutrophil phagocytosis	%	51.7 ± 11.7†‡	79.2 ± 11.5*	74.7 ± 14.6*
% lymphocytes[b]	%	29.5 ± 8.0†‡	40.1 ± 10.8*‡	53.0 ± 6.6*†

* significant *vs* sugar-free diet; † significant *vs* sucrose diet; ‡significant vs honey diet (p values given in the text)

[a] Food intake was assessed during 7 week-long periods, each two months apart. Animals were placed into mesh-bottom cages and the amount of consumed diet recorded. A 4-day lead-in period was used prior to assessing food intake to allow the animals to acclimitise to the different cages. Food intake was corrected for percentage dry matter (determined by heating duplicate samples for 16 hours at 105°C)

[b] Percentage of leukocytes that were lymphocytes. The data was generated by the flow cytometer; actual counts were not generated.

DISCUSSION

Long-term feeding of honey has been shown in our laboratory to have a number of health benefits, including improved weight regulation, decreased oxidative damage and improved cognitive functions compared with rats fed sucrose or a sugar-free diet (Chepulis and Starkey, 2007; Chepulis et al., 2007) and it appears that honey may also stimulate immune function in aged rats. Neutrophils mediate the immediate host response to bacterial and fungal

infections, which are largely responsible for the higher rates of mortality and morbidity seen in older populations (Butcher et al. 2001). In human populations, total neutrophil numbers are not thought to decline; however, a decrease in neutrophil function (phagocytic activity) has been shown to occur with increasing age (Wenisch et al. 2000; Butcher et al. 2001).

In this study, the degree of neutrophil phagocytosis was similar in both honey- and sucrose-fed rats (75–80%), both of these treatments having approximately 50% higher levels of phagocytosis than rats fed the sugar-free diet. Many other studies have investigated neutrophil function in rats, but given that every trial used different diets, breeds, age and drug interventions it is impossible to get comparable data. Thus, it is impossible to know whether the higher levels of neutrophil phagocytosis in honey- and sucrose-fed rats reflect a reduced decline with age, or whether there has been a stimulation of phagocytic activity in these two groups that did not occur in those fed the sugar-free diet. A review of the literature revealed nothing that might suggest that sucrose could affect the activity of the immune system, although there is some limited data available for the effects of feeding honey on total neutrophil numbers. In one small study (Al-Waili, 2004) 12 sheep (6–8 months; 25–30 kg) were randomized to receive either 500 ml of intravenous honey (5% w/v) or saline once every 10 days for 40 days, with blood removed at 0, 10, 20, 30, 40 and 50 days. The % neutrophils (it is not stated, but the reader must assume that the author mean the percent of leukocytes that were neutrophils) increased from 55% at time zero to 76% in the honey group after 10 days, whereas no difference was observed in the saline group. In the present study, no actual neutrophil counts were measured, but it is possible that the increased phagocytosis seen in honey- and sucrose-fed rats resulted from an increase in the number of new (and maybe more active) neutrophils. This idea is supported by the finding that the percentage of leukocytes that were lymphocytes was higher in honey- and sucrose-fed rats compared with those given the sugar-free diet (and also higher in honey-fed rats compared to those fed sucrose). Ideally, it would have been best to have measured the levels of neutrophil phagocytosis and lymphocyte counts throughout the 12 months, rather than just at the end of the study, so as to get a better idea of the trend of phagocytic activity over time; however, this was not practical in this study due to financial constraints. It would certainly be advantageous, though, to investigate these trends in lymphocyte counts and neutrophil phagocytosis in future feeding studies.

The findings from this research do demonstrate, though, that honey may have a better immuno-modulatory effect than sucrose alone, as honey-fed rats had percentage lymphocyte counts that were 1.3 times higher than those of the sucrose-fed animals. The honey used in this study contained 40.9% fructose, 30.1% glucose, 0.5% sucrose, 3.4% maltose and 5.1% oligosaccharides (Chepulis, 2007), these levels being comparable to the average values reported elsewhere (White, 1975; Rousseau et al. 1980). As sucrose is a dimer of glucose:fructose, the dietary concentrations of fructose and glucose resulting from the intake of the sucrose diet would both have been

3.95g / 100g diet. This is comparable to the 4.09g / 100g fructose and 3.01g /100g glucose consumed in the honey diet (4.34g and 3.26g /100g, respectively, when the sucrose present in honey is taken into account), suggesting that the sugar content of the two diets does not explain the difference in lymphocyte counts, although it is possible that the small differences in sugar concentrations may have been involved. Indeed, honey has been shown to have a mitogenic effect on human B and T lymphocytes (Abuharfeil et al. 1999) and it has been suggested that this may occur through the glycosylation of proteins resulting from the high sugar content (glycosylated proteins have been shown to activate a number of cell types).

Research has also demonstrated that sugars that are slowly absorbed can lead to the formation of short chain fatty acid (SCFA) fermentation products in the gut (Kruse, 1999). SCFA are commonly formed after ingestion of pre-biotic foods, and with the pre-biotic effects of honey established *in vitro* (Ustunol and Ghandi, 2001; Sanz et al. 2005) it is possible that the ingestion of honey may also result in their formation. A number of studies have demonstrated, either directly or indirectly, that SCFA can have immunomodulatory properties (reviewed by Schley and Field, 2002). Thus it is possible that honey may also be stimulating the immune system via the presence of fermentable sugars in the gut.

It is also possible that non-sugar components of the honey may be responsible for the enhanced immuno-modulatory effects seen. Tonks et al. (2007), for example, have recently reported that a 5.8 kDa component of Manuka honey is capable of stimulating immune function *in vitro*. These authors determined that the compound was not a lipopolysaccharide, amino acid, vitamin or mineral, but further investigations are required to elucidate the nature of the compound. It is also possible that the antioxidant content of the honey may have contributed to its immuno-modulatory effects. Although there are no studies directly investigating the effects of honey antioxidants on immune function, other antioxidant compounds have been shown to stimulate immune function *in vitro* (Sabongi et al. 1997) and *in vivo* (De La Fuente et al. 2002). However, further investigations, perhaps using artificial immune challenges, are recommended to better elucidate these effects.

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The Long-Term Effects of Feeding Honey Compared with Sucrose and a Sugar-Free Diet on Weight Gain, Lipid Profiles, and DEXA Measurements in Rats

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ABSTRACT: To determine whether honey and sucrose would have differential effects on weight gain during long-term feeding, 45 2-mo-old Sprague Dawley rats were fed a powdered diet that was either sugar-free or contained 7.9% sucrose or 10% honey *ad libitum* for 52 wk (honey is 21% water). Weight gain was assessed every 1 to 2 wk and food intake was measured every 2 mo. At the completion of the study blood samples were removed for measurement of blood sugar (HbA1c) and a fasting lipid profile. DEXA analyses were then performed to determine body composition and bone mineral densities. Overall weight gain and body fat levels were significantly higher in sucrose-fed rats and similar for those fed honey or a sugar-free diet. HbA1c levels were significantly reduced, and HDL-cholesterol significantly increased, in honey-fed compared with rats fed sucrose or a sugar free diet, but no other differences in lipid profiles were found. No differences in bone mineral density were observed between honey- and sucrose-fed rats, although it was significantly increased in honey-fed rats compared with those fed the sugar-free diet.

Keywords: HDL-cholesterol, honey, obesity, sugars, weight gain

Introduction

Several aspects of health are known to deteriorate as a natural part of the aging process. However, many physiological disorders that can occur in later life, including obesity, cardiovascular disease, and diabetes, are influenced both by genetics and by factors that have accumulated over the span of a lifetime. Most age-related disorders have complex etiologies and are not due to a single factor. In particular, dietary choices often have a significant impact on disease development and progression, and recent evidence suggests that diseases such as obesity, diabetes, atherosclerosis, hyperlipidemia, insulin resistance, and cognitive deterioration can be influenced by long-term dietary changes.

Overwhelming research now shows that diets containing substantial amounts of high glycemic index (GI) foods may actually be detrimental to health because of prolonged or elevated postprandial hyperglycemia. Observational studies have suggested that diets with a high glycemic load (GI × carbohydrate content) are associated with increased risks of type 2 diabetes and cardiovascular disease (Brand-Miller 2003). Similarly, the DECODE study, a meta-analysis of more than 20 studies, has shown that increased rates of mortality and morbidity are associated with high blood glucose levels and high GI diets in both diabetic and nondiabetic patients (The DECODE Study Group 1999). In addition, links have been made between a high GI diet and atherosclerosis in nondiabetic subjects (Balkau and others 1998) as well as with colon (Francheschi and others 2001; Michaud and others 2005) and breast (Augustin and others 2001) cancers. Hyperglycemia has also been shown to correlate to the development of hyperinsulinemia (excess levels of circulating insulin in the blood) and insulin resistance (Augustin and others 2002).

Low GI foods are now being suggested as a replacement for high GI foods as they induce a lower glycemic response, and this is thought to equate to a lower insulin demand, better long-term blood glucose control, and a reduction in blood lipids (Brand-Miller 2003). Epidemiological evidence suggests that low GI diets may also decrease the risk of cardiovascular disease (Liu and others 2000; Stampfer and others 2000) as well as promote satiety (because of an increase in fiber content), minimize postprandial insulin secretion, and increase fat oxidation (Liljeberg and others 1999; Augustin and others 2002). Furthermore, recent studies have also shown that dietary carbohydrate content (particularly GI) may have a large impact on weight gain as the number of individuals who are overweight or obese in the United States has increased, despite the fact that fat intake has decreased during the last 20 y (Bell and Sears 2003). It has been suggested that this is likely to be due to the high GI component in the diet, as carbohydrates in the current American diet come primarily from sugars, refined starches, and grains (Bell and Sears 2003).

With the role that postprandial glycemia can have on disease etiology, much research has been undertaken comparing the differential blood sugar response to ingestion of glucose, fructose, and sucrose (Swan and others 1966; MacDonald and others 1978; Bohannon and others 1980; Reiser and others 1986; Shambaugh and others 1990). However, there appears to be little investigation into the use of other sugars as low GI alternatives. Honey is a naturally occurring sweetener that contains a mix of both simple and complex sugars, as well as vitamins, minerals, acids, and enzymes (Molan 1996). Limited clinical studies have demonstrated

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doi: 10.1111/j.1750-3841.2007.00592.x

that honey has a lower GI than sucrose (Shambaugh and others 1990), and that honey, sucrose, and fructose do have differential effects on blood glucose levels (Samanta and others 1985; Al-Waili 2003, 2004). In addition, the beneficial effects of consuming honey are well established in the literature. These include improved antioxidant capacity (Taormina and others 2001; Gheldof and others 2003; Schramm and others 2003), enhanced gut motility (Ladas and Raptis 1999), enhanced cytokine production (Tonks and others 2003), and a prebiotic effect (Sanz and others 2005; Ezz El-Arab and others 2006). In addition, short-term feeding in rats has demonstrated that honey leads to less weight gain than sucrose after only 6 wk (Chepulis 2007).

This trial was therefore designed to ascertain what effect honey might have on weight gain, lipid profiles, and bone mineralization levels after long-term feeding, as compared with those fed a sucrose diet. A 3rd treatment group, fed a sugar-free diet, was also included in this trial as a control.

Materials and Methods

Experimental design

Fifty-five Sprague Dawley rats, aged approximately 8 wk at the start of the trial, were sourced from the small animal research facility at AgResearch, Hamilton, New Zealand. The trial was carried out in the Animal Behaviour Research Facility at Waikato Univ., Hamilton, New Zealand. The research room was maintained at 22 ± 1 °C with a 12 h reverse light/dark cycle (lights off 0700 hours). This study was approved by the Waikato Univ. Animal Ethics Committee.

Experimental diets

A honeydew honey (HD19) with a high antioxidant content (TEAC = 3.1 mmol/L) was chosen for use in this study. Three experimental diets were prepared to contain no sugar (diet nr 1), 7.9% sucrose (diet nr 2), or 10% honey (diet nr 3). All diets were prepared to contain a minimum of 5% water. An additional 21 mL of water was also added to each kilogram of the nonhoney diets to account for the fact that the honey contained 21% water (measured using a refractometer).

In this study, the diets were prepared such that they approximated the composition of a typical New Zealand diet. Based upon data from the 1997 Natl. Nutrition Survey (New Zealand Ministry of Health 1999), the diets were formulated so that of 100% total energy, 15% to16% came from protein, 35% came from fat, and 45% to 47% came from carbohydrate (CHO). In addition, skim milk powder (SMP; Fonterra Co-Operative Group Ltd., New Zealand) was added to the diets at a level of 8% of the total daily kilojoule intake. This level of SMP (8%) was chosen as it was equivalent to approximately 350 to 400 mL of milk or dairy in an average person's daily diet (assuming a total kJ intake of 11000 to 12000 per day). The SMP used in these diets had the following specifications: energy 1520 kJ/100 g, protein 36.1 g/100 g, and fat 0.8 g/100 g. A low-GI starch product (amylose) was used in the sugar-free diet as a replacement for the sucrose/honey rather than standard high-GI starch.

To enhance the possible levels of oxidative damage that may occur in these animals, the diets were prepared using used cooking oil rather than virgin oil as the source of fat. The cooking oil was sourced from various commercial kitchens in Palmerston North, New Zealand, and well mixed prior to inclusion in the diets.

Diets were prepared monthly and were kept in the dark at 4 °C or −15 °C for the duration of the study. Standard rodent vitamin and mineral mixes were prepared as described previously (Chepulis 2007); however, the mineral mix was modified to reflect the amounts of calcium (1.33 g/100 g) and phosphate (1.05 g/100 g) added to the diet from the SMP (cellulose was added instead to make up the final weight). The composition of the diets is given in Table 1.

Subsamples of the 3 diets were analyzed by the Nutrition Laboratory at Massey Univ., Palmerston North, New Zealand, for measurement of energy (bomb calorimetry). The percentage dry matter of these samples was determined monthly during the trial by drying preweighed samples (in triplicate) at 105 °C for 16 h.

Experimental procedures

Animals were weighed upon receipt, and the 5 smallest and 5 largest discarded from the trial. The remaining 45 animals were randomly allocated to 1 of 3 experimental diets and housed individually in standard rat cages with plastic bottoms and metal grid tops (final measurements 45 × 25 × 30 cm high). A 10- to 12-cm-long piece of 90-mm PVC tubing was placed into each cage to allow the animals a place to "hide." Enrichment devices (including small plastic containers, pegs, sticks, and pieces of doweling) were routinely added to the cages. Food jars containing the appropriate diet were placed into the respective cages and new diet added to the jars every 2 d so that food was available *ad libitum*. Water was freely available, and replaced twice weekly.

For assessment of food intake, the animals were removed from their home cages and placed into raised stainless steel cages that had 2 mm^2 mesh flooring. Numbered blotter papers were placed on shaving-filled trays beneath each cage to catch the spilled diet and feces and absorb the urine. The animals were allowed to acclimatize to the new cages for 4 d (animals had *ad libitum* access to their food and water but intake was not recorded) before beginning the food intake assessment. To assess food intake, food jars containing the appropriate diet were weighed and placed into each cage. New food was weighed and added to each food container daily for 7 d. On day 11, the food pots were removed and weighed. The blotter papers were allowed to dry at 25 °C for 3 to 4 d. Once dry, the spilled diet was scraped off the blotters, separated from the feces/hair and so on by passing through a 2-mm sieve and weighed.

All animals were weighed every 1 to 2 wk, and food intake was assessed every 2 mo (including the start of the trial).

Table 1—Composition of the experimental diets (g/kg).

Ingredient	Sugar-free diet	Sucrose diet	Honey diet
Skim milk powder[a]	95	95	95
Casein[b]	120	120	120
Used oil	160	160	160
Amylose[c]	79	—	—
Sucrose[d]	—	79	—
Honey	—	—	100
Cellulose[e]	50	50	50
Modified mineral mix[f]	50	50	50
Sugar-free vitamin mix[g]	5	5	5
Starch	365	365	365
Water (mL)	76	76	55

[a]Fonterra Co-Operative Group Ltd., New Zealand.
[b]80 mesh, New Zealand Milk Products.
[c]Davis Trading Co., Palmerston North, New Zealand.
[d]Sigma Chemical Co.
[e]Avicel PH102, Commercial Minerals Ltd., Auckland, New Zealand.
[f]A mixture supplying (g/kg diet) Ca 4.96, Cl 7.79, Mg 1.06, P 3.81, K 5.24, Na 1.97; (mg/kg diet) Cr 1.97, Cu 10.7, Fe 424, Mn 78.0, Zn 48.2; (μg/kg diet) Co 29.0, Mo 152, Se 151.
[g]A mixture supplying (mg/kg diet) retinol acetate 5.0, DL-α-tocopherol acetate 200.0, menadione 3.0, thiamin hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-panothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg/kg diet) ergocalciferol 25.0, cyanocobalamin 50.0.

Sample analyses

On days 364 and 365 (half of each treatment group on each day), the rats were anaesthetized using CO_2 gas. Each animal underwent a cervical dislocation before approximately 10 mL of blood was removed from the heart via cardiac puncture using 19-gauge needles. Approximately 3 mL of the removed blood was added to an EDTA blood collection tube and the remainder added to a standard vacutainer. These latter blood samples were allowed to clot before centrifuging at 3000 × *g* for 10 min and removing the serum (for fasting lipid profiles). Both the EDTA-collected blood and the serum were then analyzed at Waikato Hospital (Hamilton, New Zealand) using standard laboratory procedures for measurement of glycated hemoglobin (HbA1c) (HPLC Biomate Affinity column) and fasting lipid profiles (Roche Method using P800 Hitachi). The bodies were then stored at −18 °C for later analysis.

After approximately 3 wk in the freezer, 36 rat bodies (12 from each treatment) were sent to the Dept. of Nutrition and Health at Massey Univ., New Zealand, for DEXA analysis. Bone mineral measurements were taken using a fan beam Hologic QDR Discovery bone densitometer (Bedford, Mass., U.S.A.). A quality control (QC) scan of an anthropomorphic spine phantom was taken to ensure the unit's precision. The rat whole body was scanned using collimator size 10.24 × 0.10 with point resolution of 0.064 cm and 0.1512 cm line spacing. Regional high-resolution scans of the lumbar spine, right and left femurs were performed using 5.69 × 0.03 collimator. Point resolution and line spacing were 0.0311 cm. Rats were positioned supine with right angles between the spine and femur and between femur and tibia.

For the *ex vivo* scans the left femurs were stripped of extraneous tissue, leaving about 1 cm of flesh attached. These were submerged in a 1.5-cm-deep dish of PBS. Regional high-resolution scans of the left femurs were performed using 5.69 × 0.03 collimator. Point resolution and line spacing were 0.0311 cm.

Statistical analyses

A 1-way analysis of variance (ANOVA) was performed on all parameters to determine if there were statistical differences between the 3 dietary treatments. Where ANOVA was significant, post hoc tests using a Bonferroni correction were carried out to determine which groups differed. These results are presented below. All analyses were carried out using SPSS version 12 (SPSS, Chicago, Ill., U.S.A.).

Results

For reasons unrelated to diet (2 animals turned out to be female rather than male, and 2 animals died from a fall after escaping from their cages), 4 rats had to be excluded from the study, resulting in final numbers of 13 in the sugar-free group, 14 in the sucrose groups, and 14 in the honey group.

Weight gain and food intake

The results of this study (presented in Table 2) suggest that honey does affect weight gain in adult rats. Mean overall percentage weight gain in honey-fed rats was similar to that observed in rats fed a sugar-free diet, and was significantly reduced by 23.4% compared with those fed sucrose after 52 wk ($P = 0.015$). Overall percentage

Table 2—Endpoint data (mean ± SD) for rats fed diets that were either sugar-free, or contained 7.9% sucrose or 10% honey (honey is 21% water).

		Diet		
Result	Unit	Sugar-free (Diet nr 1)	Sucrose (Diet nr 2)	Honey (Diet nr 3)
Overall weight gain	%	102.5 ± 19.7†	130.6 ± 26.7*	107.2 ± 13.8†
Overall food intake[a,b]	g/7 w	1246.4 ± 85	1243.6 ± 111	1244.8 ± 89
Overall kilojoule intake	kJ/7 w	23182 ± 1580	23019 ± 2053	22730 ± 1620
Fecal output[b]	g/7w	131.4 ± 6.9	124.7 ± 12.6	133.1 ± 10.9
HbA1c	mmol/L	4.07 ± 0.17	4.19 ± 0.14	3.97 ± 0.12†
Total cholesterol	mmol/L	2.79 ± 0.45	2.98 ± 0.59	3.12 ± 0.37
LDL cholesterol	mmol/L	0.09 ± 0.09	0.10 ± 0.14	0.06 ± 0.14
HDL cholesterol	mmol/L	2.32 ± 0.33	2.44 ± 0.51	2.82 ± 0.30*†
Triglycerides	mmol/L	0.85 ± 0.24	0.96 ± 0.34	0.86 ± 0.33

*Significant ($P < 0.05$) compared with sugar-free diet. †Significant ($P < 0.05$) compared with sucrose diet.
‡Significant ($P < 0.05$) compared with honey diet.
HbA1c = glycated hemoglobin; HDL = high-density lipoprotein; LDL = low-density lipoprotein.
[a]Food intake corrected for percentage dry matter (determined by heating duplicate samples for 16 h at 105 °C).
[b]Data collected from the 7 wk of food intake assessment only.

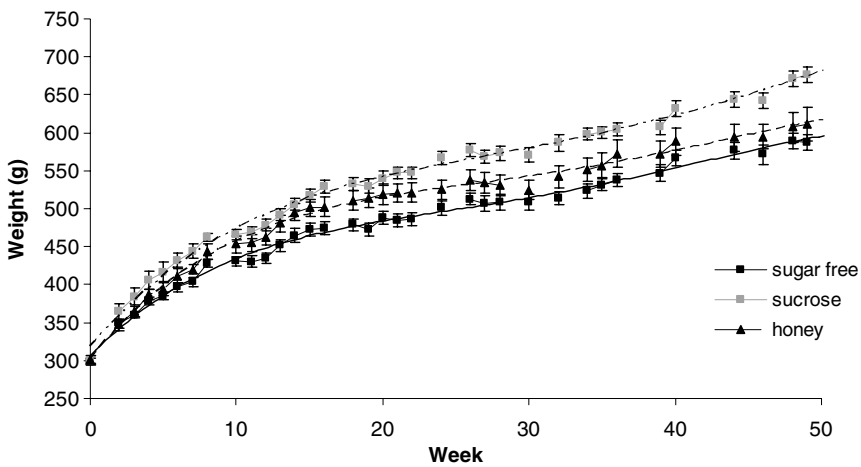

Figure 1—Actual mean weight gain (± SEM) for rats fed diets that were either sugar-free or contained 7.9% sucrose or 10% honey. All diets were formulated to contain equivalent amounts of honey/sugar/amylose and water (the honey was 21% water).

weight gain was also significantly reduced in rats fed the sugar-free diet compared with the sucrose-fed rats ($P = 0.004$). Figure 1 shows the overall percentage weight gain over the duration of the study.

Food intake was assessed during 7-wk-long periods, each measurement period being 2 mo apart. No differences were observed between treatments at any of these endpoints, or in the 7-wk actual or total kilojoule intake.

Blood sugar (HbA1c) and cholesterol levels

HbA1c levels were significantly lower in rats fed the honey diet compared with those fed the sucrose diet ($P = 0.001$), but there were no differences observed between the sucrose and sugar-free treatment groups (see Table 2). No differences in total cholesterol, LDL cholesterol, or triglyceride levels were observed after 52 wk of feeding between any of the respective treatments; however, HDL cholesterol was shown to be 16% to 21% higher in honey-fed rats than in those fed the sucrose ($P = 0.044$) or sugar-free ($P = 0.006$) diets.

DEXA scans

Full body scans by DEXA revealed a small number of differences in the body composition of the animals in the 3 dietary treatments (see Table 3). Lumbar spine area was greater in both sucrose- and honey-fed rats compared with those given a sugar-free diet (both $P < 0.05$); and whole body area ($P = 0.02$) and bone mineral composition ($P = 0.002$) were higher in rats fed sucrose compared with those given the sugar-free diet. Honey-fed rats exhibited a slight, but significant, increase in mean whole body bone mineral density (BMD) compared with those fed a sugar-free diet ($P = 0.009$).

Mean total percent body fat was higher in sucrose-fed rats (34.7%) than in honey-fed rats after 12 mo (25.5%; $P = 0.025$). No significant differences were observed between sucrose-fed rats and those given a sugar-free diet, although percentage body fat levels were similar for animals given honey and the sugar-free diet (26.5%).

Discussion

As the results from this study demonstrate, there do appear to be health benefits associated with consuming honey for a prolonged period of time. Weight gain was substantially reduced in honey-fed rats compared with those given a sucrose-based diet, and this agrees with the earlier work of Chepulis (2007) that showed that honey reduced weight gain compared with sucrose in short-term feeding. Importantly, these comparable data suggest that the weight regulating property of honey is not restricted to only young animals (animals were aged 6 to 12 wk in the earlier study) but instead may occur throughout the lifetime of the animal, regardless of the age of the individuals involved. In the earlier work (Chepulis 2007), however, reduced weight gain occurred in honey-fed rats even though the overall 6-wk food intake was significantly higher in these animals compared with those fed the sugar-free diet. In contrast, food intake in the current study appears to be similar for all treatments groups throughout the duration of the study. Food intake in the current study was assessed during 7-wk-long periods and not for the total duration of the study, as occurred in the earlier study. Due to the need to house the animals individually long term, it was not ethically viable to use food assessment cages for everyday housing. These cages are smaller than those required by the Waikato Univ. Ethics Committee, and as they have a mesh floor, they are unsuitable for long-term use. However, given the standard housing conditions used throughout the 12-mo period, it is unlikely that food intake would have varied between the 3 dietary treatments during the times that food intake was not measured. In the work by Chepulis (2007), food intake (and kilojoule intake) was significantly higher in the sucrose and honey groups compared with the sugar-free animals and it was suggested that this was due to the extra kilojoules provided by the honey or sugars in the diet. In the present study, however, kilojoule intake was the same for all 3 dietary treatments. This suggests that both the sugar-free and honey-based diets in the present study demonstrated a reduced weight gain/kJ compared with the sucrose-fed group.

It must be noted though that the sugar-free diet prepared in the current study was not entirely sugar-free as it contained nearly 10% SMP. SMP contains, on average, 52% lactose; therefore, even the sugar-free diet contained approximately 5% sugars by weight. This may have been sufficient to increase the sweetness of the diet, thereby improving palatability. An increase in palatability may account for why food intake was not reduced in rats fed the sugar-free diet in the current study, whereas it was in the trial presented by Chepulis (2007). However, a higher kilojoule intake should have led to more weight gain and clearly this was not seen in the animals

Table 3—Mean DEXA data (mean ± SD) for rats fed diets that were either sugar-free or contained 7.9% sucrose or 10% honey (honey is 21% water).

		Diet		
Result	**Unit**	**Sugar-free (Diet nr 1)**	**Sucrose (Diet nr 2)**	**Honey (Diet nr 3)**
Left femur				
Area	cm^2	1.94 ± 0.07	2.04 ± 0.11	2.07 ± 0.12*
BMC	g	0.59 ± 0.04	0.64 ± 0.07	0.65 ± 0.07
BMD	g/cm^2	0.30 ± 0.01	0.31 ± 0.02	0.31 ± 0.02
Right femur				
Area	cm^2	1.98 ± 0.13	2.02 ± 0.11	2.09 ± 0.07
BMC	g	0.60 ± 0.05	0.64 ± 0.07	0.65 ± 0.05
BMD	g/cm^2	0.30 ± 0.01	0.32 ± 0.02	0.31 ± 0.01
Lumbar spine				
Area	cm^2	2.67± 0.07	2.84 ± 0.14*	2.83 ± 0.15*
BMC	g	0.65± 0.04	0.71 ± 0.11	0.72 ± 0.07
BMD	g/cm^2	0.24 0.01	0.25 ± 0.03	0.25 ± 0.02
Whole body				
Area	cm^2	82.9 ± 5.3	90.1 ± 6.9*	85.1 ± 3.9
BMC	g	12.5 ± 0.6	14.4 ± 1.6*	13.7 ± 0.65
BMD	g/cm^2	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01*
Percent fat	%	26.5 ± 5.4	34.7 ± 9.1‡	25.5 ± 6.4†

*Significant ($P < 0.05$) compared with sugar-free diet. †significant ($P < 0.05$) compared with sucrose diet; ‡significant ($P < 0.05$) compared with honey diet.
BMC = bone mineral composition, BMD = bone mineral density.

fed the sugar-free diet. This suggests that differences in kilojoule intake are not solely responsible for the weight gains (or lack thereof) observed in this study.

Importantly, the 3 diets only differed in the content of the amylose, sucrose, and honey. Given that both amylose and honey are low GI ingredients, whereas sucrose is a high GI ingredient, these findings lend weight to the theory that glycemic index and the resultant blood sugar levels may be responsible for the reduced weight gain. These data are further supported by the finding that blood sugar (HbA1c) levels were indeed reduced in both sugar-free and honey-fed rats compared with sucrose-fed animals, although this difference did not reach significance for the animals fed the sugar-free diet. Glycated hemoglobin (HbA1c) is routinely used as a measure of long-term serum glucose regulation as it is easily quantifiable, with the hemoglobin occurring in large quantities in the blood. However, glycation is a nonenzymic, free-radical process, and the presence of antioxidants can reduce the frequency of this reaction, thereby reducing the amount of HbA1c present. The honey used in this study had a high antioxidant content and it is possible, therefore, that the antioxidant content of the honey, rather than its low GI properties, was responsible for the reduced HbA1c level detected in these animals. HbA1c levels were not reduced in honey-fed rats (nor in any of the other treatment groups) in the trial in Chepulis (2007), and it is difficult to explain why this is so, given that similar honeydew honeys with similar antioxidant contents (TEAC = 2.7 and 3.1) were used. It could be that the earlier trial simply did not run for a long enough period of time to be able to detect a difference (HbA1c is a marker of the level of hemoglobin glycation occurring over the previous 4 to 12 wk), or that other factors (either diet- or age-related) affected the level of glycation that occurred.

Certainly, the aforementioned results agree with the literature that low GI foods can improve weight regulation compared with their higher GI counterparts (Agus and others 2000; Spieth and others 2000; Dumesnil and others 2001; Brand-Miller and others 2002), and the World Health Organization has even issued an extensive report detailing the use of low GI foods as an appropriate way for preventing obesity (Joint FAO/WHO Expert Consultation 1998). However, it must be noted that fructose is metabolized through very different pathways to glucose, and the 2 sugars elicit very different hormonal responses after ingestion (Wylie-Rosett and others 2004). In particular, fructose consumption has been associated with increased lipogenesis and reduced satiety compared with glucose (Teff and others 2004), and it has been suggested that high levels of fructose intake may actually contribute to the obesity epidemic seen in Western populations rather than reduce it. Thus, the idea that honey-fed rats exhibit less weight gain than sucrose-fed rats simply because of differences in GI may not be entirely accurate. Unpublished work in our laboratory has shown that rats fed 60% honey exhibit weight gains that are lower than those fed an equivalent amount of mixed sugars (as in honey) and that weight gains are similar for animals given the sucrose and mixed sugars diets. This suggests that the difference in weight gain between sucrose- and honey-fed rats is not simply due to the different sugars present in the diet, but that other factors may be involved. As discussed in Chepulis (2007), the reduced weight gain seen in honey-fed rats may be due to the insulin-mimetic effects of hydrogen peroxide produced by the honey. No studies have been undertaken to assess whether hydrogen peroxide could reach sufficient levels *in vivo* to elicit such a response, although it warrants further investigation.

Interestingly, cholesterol parameters were also altered in this study, with honey-fed rats exhibiting HDL cholesterol levels that were 15% to 20% higher than those fed the sugar-free and sucrose diets. No other long-term feeding studies have investigated the effects of honey on lipid profiles; however, short-term animal feeding studies have shown no increases in HDL cholesterol levels, either compared with baseline levels or with other dietary treatments (Al-Waili 2004; Chepulis 2007). It is possible that the changes in HDL cholesterol observed in the present study occurred gradually over a prolonged period of time rather than occurring in only a few weeks. Research has shown that there is a strong link between high GI diets and low HDL cholesterol levels (Frost and others 1999; Luscombe and others 1999; Buyken and others 2001; Ford and Liu 2001; Liu and others 2001); thus, it is possible that the low GI of the honey diet may have contributed to the increase in HDL cholesterol levels. However, as HDL cholesterol levels were comparable between the low GI sugar-free diet and the high GI sucrose diet, it is unlikely that dietary GI played a significant role. Several factors have been reported to improve HDL cholesterol levels in humans, including aerobic exercise, weight loss, cessation of smoking, and supplements such as omega 3, monounsaturated fat, and nicotinic acid (Drexel 2006), although it is hard to see how any of these factors may be relevant to the current study, except maybe the weight loss. Van Gaal and others (1997) reported that a weight reduction of as little as 5% to 10% can significantly improve HDL cholesterol levels in overweight individuals. Similarly, several authors have reported specific improvements in HDL cholesterol levels in both men and women after 12 to 18 mo of weight-reducing diets (Wood and others 1991; Williams and others 1994; Wing and Jeffery 1995; Stefanick and others 1998). It is possible, therefore, that the increase in HDL cholesterol levels seen in honey-fed rats was directly related to the reduced levels of body weight compared with animals fed the sucrose diet. However, if the improvement in HDL cholesterol was purely due to reduced body fat levels, then animals fed the sugar-free diet should also have demonstrated increases in HDL cholesterol levels compared with sucrose-fed animals, and this did not occur. Thus, it would seem that there are other factors responsible for the improvement in HDL cholesterol levels observed in honey-fed rats.

It is important to note, though, that rodents are generally not an appropriate model to assess lipoprotein concentrations in blood as plasma lipid levels are only minimally affected by modifications of dietary fat and cholesterol compared with their response in humans (Hegsted 1975). Indeed, it has been suggested that guinea pigs may be a better model for cholesterol and lipoprotein metabolism (Fernandez 2001). However, the data reported in the present study were a part of a larger investigation that assessed the effects of honey, sucrose, and the sugar-free diet on cognition (Chepulis and Starkey 2007), and rats are a well-accepted model for behavioral endpoints (Ingram and others 1994; Benton and others 2003).

The finding that consuming honey increases HDL cholesterol levels is still a significant result though. In clinical trials, there have been strong associations seen between low HDL cholesterol levels and the increased risk of cardiovascular disease (Drexel 2006). Several large studies (Carlson and Rosenhammer 1988; Rubins and others 1999; Canner and others 2003) have also investigated the use of HDL-cholesterol-raising agents as a therapeutic strategy for improving cardiovascular outcomes in high-risk populations, and the findings have been impressive. The risk of death, myocardial infarction, stroke, or revascularization was reduced by up to 90% with only minimal improvements in HDL cholesterol levels. The ability to improve HDL cholesterol through simple dietary means is therefore a valuable tool, particularly in individuals that are high risk for the development of cardiovascular disease.

Few differences between treatments were found in the bone-related DEXA measurements performed in this study, and this agrees with unpublished work from our laboratory that honey does not improve bone calcium levels during normal feeding regimes. Certainly, no differences in any of the DEXA endpoints were observed between rats fed sucrose and honey, suggesting that the type of sugars ingested long term may have little impact on bone density or mineralization levels. No other long-term data are available for the effects of honey on bone density measures, but evidence suggests that long-term feeding of high sucrose diets can alter the calcium balance in humans (Lemann and others 1970; Thom and others 1978; Ericsson and others 1990) and negatively affect bone mineralization levels (Li and others 1990; Saffar and Markis 1992; Salem and others 1992). Such decreases in bone density and mineralization were not seen in sucrose-fed rats in the present study, but it may be that the levels of sugars were not high enough to induce reductions in bone strength and density (sugar levels in the aforementioned studies were up to 65%). In addition, the addition of SMP to the diets in this current study may have aided calcium absorption in all 3 dietary treatments due to the presence of casein and the possible formation of casein phosphopeptides in the gut.

Conclusions

Honey appears to have a number of health benefits associated with long-term feeding, including improved weight regulation and reduced blood glucose levels as well as increased HDL-cholesterol levels. These effects may result from differences in GI compared with sucrose and because of its antioxidant content, although other factors may also be involved. Honey may therefore be an effective replacement for sucrose in individuals who suffer from poor glycemic control or who are high risk for CHD.

Acknowledgments

This study was funded by Fonterra Brands Ltd. New Zealand as a part of a PhD program. The authors would also like to thank Dr. Peter Molan of Waikato Univ. for his assistance.

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